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The association of adrenaline and noradrenaline with blood platelets. By H. Weil-Malherbe and A. D. Bone

Spectral methods of characterizing anthocyanins. By J. B. Harborne

A sensitive method for estimation of oxaloacetate. By G. Kalnitsky and D. F. Tapley

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3. Comparison of enzymic catalysis by acid phosphatase with non-enzymic catalysis at acid pH values. By R. K. Morton

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Thetin–homocysteine transmethylase. Some further characteristics of the enzyme from rat liver. By G. A. Maw

Further observations on the oxidation of di-iodotyrosine derivatives. By Rosalind Pitt-Rivers and A. T. James

THE BIOCHEMICAL JOURNAL

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CORRIGENDUM

The biochemistry of organotin compounds
Trialkyltins and oxidative phosphorylation

By W. N. Aldridge

Volume 69 (1958)

p. 368, col. 1, line 9

after EDTA (0·001 M) *insert* , glycylglycine (0·0167 M)

Metabolism of Xylose by the Lens

CALF LENS *IN VITRO*

By RUTH VAN HEYNINGEN
Nuffield Laboratory of Ophthalmology, University of Oxford

(*Received* 14 *January* 1958)

When a 21-day-old rat is fed with a diet containing more than 20% of D-xylose, complete opacity of the lens (cataract) results within about 3 weeks (Mitchell, Merriam & Cook, 1937; Darby & Day, 1939, 1940; Sterling & Day, 1951; Booth, Wilson & DeEds, 1953). Loss of weight, diarrhoea and abdominal distension also result from feeding with this diet. If the diet is not begun until the rat is 40 days old, no cataract occurs (Booth *et al.* 1953).

The mechanism whereby xylose brings about cataractous changes in the lens is unknown. The feeding of galactose (Mitchell & Dodge, 1935) or the raising of blood glucose in experimental diabetes (Chaikoff & Lachman, 1933; Patterson, 1953) also causes cataract in young rats. It has been believed that all three sugars act by means of a similar mechanism directly on the lens, by virtue of their elevated levels in the aqueous humour, but the evidence is conflicting (Patterson, 1955).

It was decided therefore to investigate the effect of one of the sugars, xylose, on the lens, first *in vitro* and later *in vivo*, in the hope of elucidating the metabolic changes within the lens. Experiments on galactose cataract in young rats have shown the accumulation of galactose 1-phosphate in the epithelial cells just beneath the lens capsule (Schwarz & Golberg, 1955).

D-Xylose appears to be only slightly metabolized by rats (Blatherwick *et al.* 1936; Miller & Lewis, 1932). Evidence that xylose can be used to some extent in the formation of glycogen in the liver of rats has recently been obtained (Hiatt, 1957). The pathways of xylose metabolism in the animal body are not known, and its phosphorylation by animal tissues has not been demonstrated. Xylulose has been shown to be phosphorylated in the liver to form xylulose 5-phosphate (Hickmann & Ashwell, 1956), and thence it can be metabolized by way of the pentose phosphate cycle. In some bacteria xylose has been shown not to be phosphorylated directly, but converted first into xylulose (Hochster, 1955; Stumpf & Horecker, 1956; Kline & Baron, 1957) and thence into xylulose 5-phosphate. In *Pseudomonas* xylose is rapidly converted into xylonic acid (Lockwood & Nelson, 1946), but the mechanism was not examined.

Only the very young rat is susceptible to xylose cataract; its lens is small (20 mg.) and therefore yields very little experimental material, and it also is difficult to remove without damage. However, the lens of the calf is relatively easy to remove from the eye without perceptible damage and is available in sufficient quantity. It has the further advantage that a considerable amount of information about its acid-soluble phosphates is available (Klethi & Mandel, 1957; van Heyningen & Pirie, 1958). It was decided therefore to make preliminary investigations on the calf lens *in vitro* before investigating the metabolism of the rat lens. The calves were usually about a week old when killed.

In this work evidence of the formation of xylulose and of phosphorylated intermediates of xylose metabolism was sought but not found. Instead, it has been shown that xylose is oxidized to xylonic acid by the intact calf lens *in vitro* by means of a dehydrogenase reducing diphosphopyridine nucleotide (DPN).

EXPERIMENTAL

Methods

Lens dispersions. Calf lens was ground in a mortar until a rather thick smooth gruel was obtained.

Samples (1–2 g.) of this dispersion were poured into weighed centrifuge tubes and the weights of the samples determined by reweighing. Small volumes of concentrated solutions of the substances with which the lens was to be incubated were added to the dispersions to give the desired final concentration and the mixtures stirred with a glass rod, which was then left in the tube. Thus the lens dispersion was scarcely diluted. When glucose was to be present in all the experimental tubes at the same concentration it was added to the dispersion in the mortar. The open tubes were incubated in a water bath at 37°. To stop the reaction ice-cold trichloroacetic acid was added, usually 5 vol. of 6% (w/v), and after a thorough stirring the rod was removed and the mixture centrifuged at 5000 *g* for 10 min. The extracts were poured off, and analysed at once or stored frozen. When phosphorus compounds were to be analysed, precautions were taken to keep the mixtures as cold as possible after the addition of trichloroacetic acid and centrifuging was at 0° at 10 000 *g* for 15 min.

Lens extracts for measurement of enzyme activity in a Beckman spectrophotometer. Calf lens was ground in a mortar with twice its weight of water and the dispersion

was dialysed overnight at 4°, against deionized water or 0·9% NaCl. The insoluble part was removed by centrifuging.

Incubation of intact lens. Calf eyes were removed as soon as possible after death and brought to the laboratory at the ambient temperature. With as little handling as possible, and without touching the front surface (on which the epithelium is located), the lens was removed from the eye and placed, front surface upwards, on a watch glass. It was then at once gently slid into an open vessel containing Krebs–Ringer phosphate solution (Umbreit, Burris & Stauffer, 1949), with suitable additions, at 37°, so that it rested on its back surface and was completely covered with fluid. The open vessels were incubated in a water bath at 37° and occasionally gently moved. When lactic acid formation, penetration of xylose or glucose or use of xylose was to be measured, each lens was incubated in a separate vessel (usually with 10 ml. of fluid) and the two lenses from one animal were compared. For the chromatographic analysis of extracts after incubation several lenses (five to ten) were incubated together in a beaker of diameter 7·5 cm. and height 7·0 cm., in a xylose-containing medium, and the same number in a medium without xylose. All the lenses lay on the bottom of the beaker and there was plenty of room for free circulation of the fluid. Usually lenses were incubated in Ringer solution containing 1 mg. of D-glucose/ml., with or without the addition of 4 mg. of D-xylose/ml. On one occasion (see Table 4) 5 mg. of D-xylose/ml. was added. The volume of fluid was about 10 ml./lens.

After incubation the lenses were quickly washed in several changes of deionized water and 5 vol. of ice-cold 6% (w/v) trichloroacetic acid was added. The lenses were disintegrated with a glass rod, and the mixture was chilled for 30 min. at 4° and then centrifuged for 15 min., 12 000 *g* at 0°. The extracts were poured off and analysed at once or stored frozen.

For the measurement of lactic acid, which diffuses into the medium during incubation, the lens plus medium was precipitated with 10 ml. of 10% (w/v) trichloroacetic acid, or else the fluid was poured off and analysed separately.

For the measurement of xylose or glucose, precipitation with $Ba(OH)_2$ and $ZnSO_4$ was used (Somogyi, 1945) to remove phosphorylated pentoses and most reducing substances. Usually the lens was ground with 10 ml. of water and 1 ml. of 0·3N-$Ba(OH)_2$ solution; 1 ml. of 5% (w/v) $ZnSO_4,7H_2O$ solution was added and after thorough shaking the mixture was filtered through hardened filter paper (Whatman no. 42).

Preparation of lens extracts for paper chromatography and electrophoresis. Trichloroacetic acid extracts were shaken three times with 3 vol. of ether to remove most of the acid. They were then treated in one of several ways. (i) Extracts were neutralized with dil. aq. NH_3 soln. and washed through a column of Amberlite IR-120 (H), 1 cm. in diam. and of length dependent on the volume of extract, neutralized with dil. aq. NH_3 soln., concentrated *in vacuo*, evaporated to dryness in a desiccator and made up to a suitable volume with water. (ii) Extracts were brought to pH 8·2 with dil. aq. NH_3 soln. and fractionated according to LePage (1949) into 'barium salts insoluble at pH 8·2', 'barium salts soluble at pH 8·2 but insoluble in 80% (v/v) ethanol' and 'barium salts soluble at pH 8·2 and in 80% (v/v) ethanol'. Each fraction was treated with Amberlite IR-120 (H). The fraction containing insoluble barium salts was shaken with several lots of the resin in a small volume of water until no more would dissolve. The combined acid extract and washings were neutralized and concentrated as above. The fraction containing barium salts insoluble in 80% (v/v) ethanol was dissolved in water and passed through a column of the resin as above. For investigation of sugars a neutral solution of the fraction containing barium salts soluble in 80% (v/v) ethanol was passed through a mixed column of Amberlite IR-120 (H) and IR-4B (OH). The neutral effluent was concentrated as above. (iii) The unneutralized trichloroacetic acid extract was washed through a column of Amberlite IR-4B (OH) and the effluent concentrated as above. The effluent contains phosphorylcholine (PC), phosphorylethanolamine (PE), glycerophosphorylcholine (GPC) and glycerophosphorylethanolamine (GPE) (van Heyningen & Pirie, 1958).

Preparation of extracts for estimation of periodate-labile phosphate of α-glycerophosphate. A water solution of the fraction containing 'barium salts soluble at pH 8·2 but insoluble in 80% (v/v) ethanol' from about 3 g. of lens (see above) was made 0·05N with respect to HCl. It was shaken with successive amounts of Amberlite IR-4B (OH) until the pH was about 6. The α-glycerophosphate (αGP) is absorbed on the resin and GPC and GPE remain in solution. The resin was washed with water and the total αGP was then eluted with three successive lots of 5 ml. of aq. 3N-NH_3 soln. Periodate-labile P was estimated in the eluate by the method of Olley & Blewett (1950). αGP, GPC and GPE are the only periodate-labile phosphate compounds present in calf lens in appreciable quantities (van Heyningen & Pirie, 1958).

Paper chromatography of lens extracts. Descending chromatography on Whatman no. 1 paper was used. The paper was washed in 2N-acetic acid, followed by six successive changes of water. The following solvents were used: Phenol–water (100:28, w/v). Butanol–propionic acid–water [prepared fresh by mixing equal volumes of *A* and *B* (*A*, 1246 ml. of butan-1-ol, 84 ml. of water; *B*, 620 ml. of propionic acid, 790 ml. of water); Benson *et al.* 1950]. Benzene–butan-1-ol–pyridine–water (1:5:3:3, upper phase; Hough, 1950). *iso*Butyric acid–aq. N-NH_3 soln. (100:60; Krebs & Hems, 1953). Propan-1-ol–aq. NH_3 soln. (sp.gr. 0·880)–water (60:30:10; Hanes & Isherwood 1949).

Paper electrophoresis. Horizontal strips of Whatman no. 3MM paper between sheets of polythene and glass were used (Kunkel & Tiselius, 1951). The potential difference along the paper was about 10 v/cm. Pyridine–acetic acid buffer, pH 4, was used (50 ml. of acetic acid, 15 ml. of pyridine and 2·5 l. of water; Grassmann, Hannig & Plöckl, 1955).

Detection and determination of substances after chromatography and electrophoresis. Phosphates were visualized by dipping the papers in the molybdate reagent of Burrows, Grylls & Harrison (1952) and, after drying, exposing the papers to sunlight or to ultraviolet light. P was estimated quantitatively by ashing the paper according to the method of Hanes & Isherwood (1949) and determining the resulting orthophosphate (P_i) by the method of Berenblum & Chain (1938). Nucleotides were located on the chromatograms by their ultraviolet absorption with a Hanovia Chromatolite (Hanovia, Ltd., London) and their positions ringed in pencil.

The $AgNO_3$–NaOH method of Trevelyan, Procter & Harrison (1950) was used to detect carbohydrates which react under these conditions. Aniline phthalate (Partridge, 1949) was used to detect aldoses, orcinol–trichloroacetic acid (Klevstrand & Nordal, 1950) to detect heptuloses, and a combination of the two sprays to detect xylulose (Hochster, 1955). Lactones were detected by the hydroxylamine method of Abdel-Akher & Smith (1951).

Other estimations. Lactic acid was measured by the method of Barker & Summerson (1941), glutathione by the method of Grunert & Phillips (1951) and pentose by that of Barrenscheen & Peham (1941). Ascorbic acid was estimated by titration with 2:6-dichlorophenol-indophenol.

Glucose (or reducing sugar) was measured by the method of Nelson (1944). To measure glucose in the presence of xylose, the $Ba(OH)_2$–$ZnSO_4$ extracts were estimated by this method before and after removal of the glucose by yeast fermentation (King & Wootton, 1956). Standards of glucose and xylose were included. The values for xylose were checked by the method of Barrenscheen & Peham (1941).

Phosphate was estimated by the methods of Fiske & Subbarow (1925) and Berenblum & Chain (1938).

Materials

D-Xylose and D-glucose were obtained from British Drug Houses Ltd., and triose phosphate acid esters (dihydroxyacetone phosphate and glyceraldehyde phosphate sodium salts, each approx. 0·02 M) from C. F. Böhringer und Söhne G.m.b.H., Mannheim-Waldhof, Germany. D-Xylulose was kindly given by Dr J. S. Fewster; D-xylonic acid was made from D-xylose by way of the cadmium bromide double salt of cadmium xylonate (Elsner, 1935).

RESULTS

Effect of xylose on the formation of lactic acid by lens dispersions

About 70% of the glucose used by rabbit lens maintained in tissue culture is converted into lactic acid (Merriam & Kinsey, 1950; Wachtl & Kinsey, 1957). Measurement of lactic acid formed by the lens under various circumstances may therefore give a guide to the normality of its metabolism. Table 1 shows the formation of lactic acid by dispersions of calf lenses, in the presence or absence of glucose and xylose; xylose is not converted into lactic acid by lens dispersions, but does not prevent the formation of lactic acid from glucose. The small amount of lactic acid formed in the absence of added glucose comes from the glucose originally present in the lens. Table 2 shows that, although no lactic acid is formed, xylose is metabolized by the lens dispersions (about 0·1 mg. used/hr./g. of lens at 37°). This disappearance of xylose is inhibited by 25 mM-iodosobenzoate and 0·1 M-iodoacetate but unaffected by fluoride or anaerobiosis. Lower concentrations of the inhibitors were not tried, since the undiluted dispersion contains much protein and glutathione thiol, with which they will also react.

Table 1. *Lactic acid formation in lens dispersions in the presence and absence of glucose and xylose*

For preparation of dispersions see Methods. Incubations in air at 37°.

Length of incubation (hr.)	Additions (mg./g. of lens)		Lactic acid (mg./g. of lens)	
	Glucose	Xylose	Found	Increase on incubation
0	0	0	0·97	—
2·5	0	0	1·21	0·24
2·5	1·3	0	1·87	0·90
2·5	1·1	1·2	1·85	0·88
2·5	0·9	0·7	1·95	0·98
2·5	0	0·9	1·20	0·23
0	0	0	0·93	—
1·5	0	0	1·06	0·13
1·5	20	0	1·81	0·88
1·5	20	30	1·83	0·90
1·5	0	30	1·10	0·17

Table 2. *Use of xylose by dispersions of lens*

Xylose (10 µl.; 10%, w/v) was added/g. of dispersion (for preparation of dispersions, see Methods). Incubation in air (unless stated otherwise) for 3 hr. at 37°. A different lens dispersion was used in each experiment.

Inhibitors	Decrease in xylose (mg./g. of lens)
None	0·22
NaF (0·1 M)	0·23
Sodium iodosobenzoate (25 mM)	0·11
None	0·33
None (in N_2)	0·38
None	0·39
Sodium iodosobenzoate (0·01 M)	0
Sodium iodoacetate (0·1 M)	0·22

Effect of xylose on the phosphorylation of glucose by lens dispersions

The phosphorylation of glucose by adenosine triphosphate (ATP) in lens dispersions, in the presence and absence of xylose, was determined by measuring decrease in pyrophosphate P of ATP (Table 3). The incubation mixture was based on that used by Long (1952). The table shows that xylose does not interfere with the phosphorylation of glucose, but is not itself phosphorylated to form a compound stable to hydrolysis for 10 min. in N-HCl at 100°.

Lactic acid formation by and penetration of sugars into intact calf lens in vitro

Table 4 shows that, with the intact lens, unlike the dispersion, the presence of xylose inhibits the formation of lactic acid from glucose. It also shows that xylose penetrates into the lens and that a relative decrease in the concentration of glucose within the lens is brought about by the presence of xylose in the incubating fluid.

Table 3. *Effect of xylose on the phosphorylation of glucose by lens dispersion*

Mixture A contained 2 g. of lens dispersion, KF (0·05 M), $MgCl_2$ (0·005 M), KCl (0·042 M) and ATP (0·004 M approx.), final vol. 6 ml. In each tube, 1 ml. of mixture A was incubated in a final vol. of 1·2 ml., with sugars added as shown below. Reaction was stopped by addition of 5 ml. of 5% (w/v) trichloroacetic acid. P_i was estimated before and after hydrolysis in N-HCl for 10 min. at 100°; difference gives value of the pyrophosphate P of ATP.

Concn. of sugar in final incubation mixture (mg./ml.)	Incubation time (hr.)	Pyrophosphate P of ATP (μg. of P)	Decrease on incubation
1. None	0	116	—
2. None	1·5	116·5	0
3. Glucose 0·4	1·5	89	27
4. Xylose 6·0	1·5	116	0
5. Glucose 0·4 + xylose 6·0	1·5	85	31

Table 4. *Penetration of xylose and glucose into the intact calf lens and formation of lactic acid in the presence and absence of xylose*

Pairs of calf lenses were incubated at 37°, each lens in 10 ml. of Krebs–Ringer phosphate solution containing 1 mg. of glucose/ml. To the incubating fluid of one lens of each pair 5 mg. of xylose/ml. was added.

Time of incubation (hr.)	Xylose (mg./g. of lens)	Glucose (mg./g. of lens)	Lactic acid in lens plus incubating fluid (mg./g. of lens)	Change due to presence of xylose in medium (mg./g. of lens): Glucose in lens	Change due to presence of xylose in medium (mg./g. of lens): Lactic acid formation
0	0	0·31	1·02	—	—
	0	0·33	0·97		
1·0	0	0·50	1·70	−0·07	−0·10
	0·87	0·43	1·60		
3·75	0	0·53	1·96	−0·12	−0·31
	1·37	0·41	1·65		
4·5	0	0·42	2·29	−0·08	−0·45
	1·36	0·34	1·84		
5·25	0	0·40	2·36	−0·23	−0·55
	1·23	0·17	1·81		
6	0	0·36	2·63	−0·21	−0·60
	1·48	0·15	2·03		

Table 5. *Lactic acid of intact calf lens incubated in the presence and absence of xylose*

One lens of a pair was incubated in 10 ml. of medium containing no glucose (see Methods) and the other in 10 ml. of the same medium containing 4 mg. of xylose/ml. Incubations were for 2 hr. at 37°. The initial level of lactic acid in calf lens is about 0·93–1·03 mg./g. of lens.

	Lactic acid (mg./g.)
No additions	1·10
With xylose	1·01
No additions	1·15
With xylose	1·15

The fact that no lactic acid is formed from xylose is shown by the finding that in the absence of glucose in the medium the amount of lactic acid formed is approximately the same whether xylose is present or not (Table 5). Presumably, in the absence of added glucose, the small amount of lactic acid comes from the glucose initially present in the lens.

The intact lens, like the lens dispersions, was shown to metabolize xylose. When a lens was incubated in 5 ml. of the incubating fluid containing 0·5 mg. of xylose for 5 hr. at 37°, analysis showed that 0·135 mg. of xylose had disappeared. The concentration of xylose used in this experiment (0·1 mg./ml.) was much lower than in the standard experimental conditions (4 or 5 mg./ml.), in order that a decrease could be detected.

The above experiments showed that xylose was metabolized by the lens. The finding that the presence of xylose inhibited the penetration of glucose into the intact lens and caused a diminution of production of lactic acid led to a decision to investigate more fully the carbohydrate and phosphate metabolism of the intact lens in the presence and absence of xylose.

Table 6. *Analysis of phosphates in calf lens after incubation of intact lens in a medium containing glucose, or glucose plus xylose*

Five to ten lenses were incubated at 37° for 4–5 hr. in medium containing glucose (1 mg./ml.) and the same number in medium containing glucose (1 mg./ml.) plus xylose (4 mg./ml.) (see Methods). Trichloroacetic acid extracts of the lens were examined for total P, P_i, P contained in compounds which are hydrolysed in 10 min. at 100° in N-HCl to form P_i and P contained in compounds not hydrolysed under these conditions.

Presence of xylose in medium	Total P (mg./g. of lens)	Difference due to xylose	P_i (mg./g. of lens)	Difference due to xylose	P hydrolysed by HCl (mg./g. of lens)	Difference due to xylose	P not hydrolysed by HCl (mg./g. of lens)	Difference due to xylose
–	0·66	–0·01	0·10	0	0·22	–0·04	0·34	+0·03
+	0·65		0·10		0·18		0·37	
–	0·68	–0·09	0·16	–0·01	0·34	–0·18	0·18	+0·10
+	0·59		0·15		0·16		0·28	
–	0·71	0	0·12	0	0·18	–0·09	0·41	+0·09
+	0·71		0·12		0·09		0·50	

Table 7. *Fractionation of calf lenses after incubation in the presence or absence of xylose*

Two lenses were incubated in medium containing glucose (1 mg./ml.) and two in the same medium containing glucose (1 mg./ml.) plus xylose (4 mg./ml.) (see Methods). Trichloroacetic acid extracts of the lenses were partitioned into three fractions with barium and ethanol (see Methods).

Incubated in	Barium salts insoluble at pH 8·2 (mg. of P)	Barium salts soluble at pH 8·2 but insoluble in 80% (v/v) ethanol (mg. of P)	Barium salts soluble at pH 8·2 and soluble in 80% (v/v) ethanol (mg. of P)
Glucose	0·72	0·39	0·16
Glucose + xylose	0·42	0·66	0·14
Difference due to presence of xylose	–0·30	+0·27	–0·02

Phosphates in lens incubated in glucose plus xylose

Table 6 shows the results of several analyses of trichloroacetic acid extracts of lens, after incubation of whole lenses in a medium containing glucose, or glucose plus xylose. It shows that the presence of xylose does not affect the amount of P_i in the lens, but brings about a decrease in the amount of P in compounds which are hydrolysed in N-HCl for 10 min. at 100° to form P_i, and a corresponding increase in phosphate compounds not liberating P_i under these conditions. The former consists almost entirely of the labile phosphate groups of the di- and tri-phosphates of nucleotides, chiefly adenosine and uridine tri- and di-phosphates (ATP, UTP, ADP, UDP) (van Heyningen & Pirie, 1958).

Analysis of lenses fractionated with barium and ethanol (Table 7) (see Methods) showed that the presence of xylose caused a relative decrease in the fraction containing barium salts insoluble at pH 8·2, thus confirming the loss of nucleotide di- and tri-phosphates, since these, together with P_i, constitute almost the whole of this fraction (van Heyningen & Pirie, 1958). The table also shows an increase in the fraction containing barium salts insoluble in 80% (v/v) ethanol, and it is in this fraction therefore that the 'difficultly hydrolysable' compound or compounds formed as the result of incubation in xylose is to be found. The monophosphates of pentoses, hexoses and sedoheptuloses would occur in this fraction if present as a result of the phosphorylation of xylose (Hochster, 1955). It has already been shown that normal calf lens contains only trace amounts of these substances (van Heyningen & Pirie, 1958).

Chromatography of fraction containing barium salts insoluble in 80% (v/v) ethanol

α-Glycerophosphate. Amounts of extract containing about 10 μg. of P were chromatographed in phenol–water and the paper was developed for the detection of P-containing compounds. Preparations from lens incubated with glucose or glucose plus xylose showed only two spots, P_i (R_F about 0·18) and a spot of R_F about 0·36. In unincubated lens this spot has been identified with αGP, which accounts for about 5·5% of the acid-soluble P in calf lens (van Heyningen & Pirie, 1958). When spots of authentic αGP were superimposed on spots of the lens extracts, the compound in the lens and the αGP did not separate after paper electrophoresis and chromatography in several solvents (phenol–water; butan-1-ol–propionic acid–water; propanol–aq. NH_3 soln.–water).

On one occasion P was estimated in the spots corresponding to αGP after paper electrophoresis at pH 4 and values of 80 μg. of P/g. of lens incubated in glucose alone, and 160 μg. of P/g. of lens incubated in glucose plus xylose, were obtained. Estimation of periodate-labile P of αGP (see Methods), in another experiment, gave values of 48 μg. of P/g. and 93 μg. of P/g. respectively for lenses incubated in the absence and presence of xylose.

Other phosphates. If more extract (20–30 μg. of P) were chromatographed in phenol–water, a spot of R_F 0·9 (PC and GPC) was seen in extracts from both types of incubation, and also a spot corresponding in position to adenylic acid (AMP). Estimation of P showed that concentrations of PC and GPC in the lenses was approximately the same whether xylose was present or not.

Spraying of trichloroacetic acid–orcinol on chromatograms of these extracts in phenol–water or in butanol–propionic acid–water showed areas in the position expected of sedoheptulose monophosphate (Benson *et al.* 1950). The spots were faint, possibly stronger in the extracts from xylose than in those from glucose incubation, and their P content was not measured as they overlapped in position with other P-containing compounds. Overspraying with aniline phthalate did not show up the presence of any xylulose phosphate or ribulose phosphate and, on heating the papers, no evidence of the presence of aldose phosphates appeared.

Capsule plus epithelium. Schwarz & Golberg (1955) in experiments on galactose cataract in the rat were able to detect the formation of galactose 1-phosphate by examination of the capsule plus epithelium. Therefore, in a further search for phosphorylated derivatives of xylose the capsules and epithelia were removed from lenses after incubation at 38° for 5 hr. in medium containing glucose and glucose plus xylose. Ten lenses were used in each group. The fraction containing 'barium salts insoluble in 80% (v/v) ethanol' was prepared and examined by chromatography, followed by treatment with the appropriate sprays. There was no trace of phosphorylated aldoses, ketopentoses or sedoheptuloses.

Non-phosphorylated compounds. Treatment of the chromatograms with alkaline silver nitrate showed two faint areas common to both extracts, glucose-incubated and (xylose plus glucose)-incubated. One of these spots had R_F 0·15 in phenol–water and has not been identified; the other, R_F 0·22 in phenol–water, corresponds to inositol, and was of equal strength in the two extracts. A third spot, very large and dark and coming up slowly after treatment with NaOH, appeared only in the extracts prepared from lenses incubated in the xylose-containing medium. An intense spot due to the presence of this compound was found in extracts of capsule plus epithelium from lenses incubated in the xylose-containing medium. This compound was found to be D-xylonic acid.

Identification of xylonic acid

This compound was detected by means of its positive reaction with $AgNO_3$–NaOH (Trevelyan *et al.* 1950). Its presence was shown either in trichloroacetic acid extracts after extraction with ether, or in the fraction containing barium salts insoluble in 80% (v/v) ethanol. Paper electrophoresis of these fractions at pH 4 showed the compound to be an acid, with a mobility of 0·62 relative to P_i (1·00). αGP had a relative mobility of 0·79.

The compound had the same R_F as synthetic xylonic acid in several solvents (Table 8), and the same electrophoretic mobility at pH 4.

The rate of formation of D-xylono-γ-lactone from synthetic D-xylonic acid was measured. The acid was treated with N-HCl at 100° and, at 15 min. intervals, samples (5 μl.) were removed for chromatography in phenol–water. The relative concentration of acid and lactone was judged by the intensity of the spots developed in $AgNO_3$–NaOH. It was found that about 50% of the acid was converted into lactone in 2 hr. Confirmation of the formation of lactone was given by means of the hydroxylamine–$FeCl_3$ spray for lactones and esters (Abdel-Akher & Smith, 1951). The compound formed from xylose by calf lens behaved in the same way as the synthetic acid, about 50% being converted in 2 hr. at 100° in N-HCl into a compound having the same R_F as the lactone formed from the synthetic acid. Table 8 gives the R_F of D-xylose, D-xylonic acid and D-xylono-γ-lactone in several solvents.

Table 8. *R_F values for* D-*xylose,* D-*xylonic acid and* D-*xylono-γ-lactone in various solvents*

Descending chromatography on acid-washed Whatman no. 1 paper was used (see Methods for details about solvents).

Solvent	D-Xylose	D-Xylonic acid	D-Xylono-γ-lactone
Phenol–water	0·46	0·29	0·62
Butan-1-ol–propionic acid–water	0·33	0·33	0·48
Benzene–butanol–pyridine–water	0·42	0·10	0·73
*iso*Butyric acid–aq. NH_3 soln.–water	0·50	0·40	0·59

Oxidation of xylose by dialysed lens extracts

Extracts of mammalian liver have been shown to oxidize xylose (Wainio, 1947) and to reduce diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) (Strecker & Korkes, 1952; Brink, 1953). Glucose was oxidized by the same systems at a faster rate; the optimum pH for the reaction was about 8. Very high concentrations of glucose are needed for the oxidation to proceed at its maximum rate, the Michaelis constant being about 37 mM at pH 7·8. The primary product of the reaction, with glucose as substrate, is D-glucono-δ-lactone, which, in alkaline solution, is converted into D-gluconic acid. The appearance of xylonic acid in lenses incubated in a xylose-containing medium suggested that a similar enzyme occurs in the calf lens.

Fig. 1 shows that at concentrations of 0·44 M the rate of reduction of DPN by dialysed lens extract (see Methods) was nearly three times faster with xylose as substrate than with glucose. When the concentration of the sugars was reduced to 0·02 M, the rate of reduction of DPN with xylose as substrate was not much decreased, but the reaction with glucose was greatly reduced, to about one-tenth of the rate with xylose. Xylose (20 mM, i.e. 3 mg./ml.) corresponds approximately to the level of sugars found in the blood of rats fed with a diet of 35% xylose (Sterling & Day, 1951). In another experiment separate extracts of the cortex and the nucleus of calf lens were made, and these were shown to contain almost the same concentration of the enzyme. An attempt was made to measure the activity of the epithelium plus capsule of lens, but material from three lenses did not contain measurable activity, thus showing that the enzyme is not greatly concentrated in the epithelium. The enzyme did not reduce TPN in the presence of either xylose or glucose.

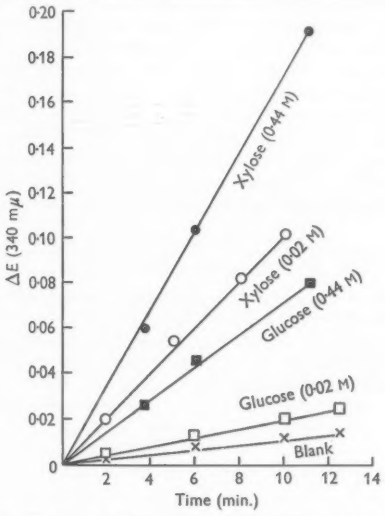

Fig. 1. Xylose dehydrogenase in calf lens; xylose and glucose as substrates. Beckman cells contained 1 ml. of dialysed lens extract, 2 ml. of phosphate buffer (M, pH 8) containing xylose or glucose (1320 or 60 μmoles), and DPN (approx. 0·2 μmole). Sugars were omitted from blank.

It appears from Fig. 2 that a single enzyme is responsible for the oxidation of both glucose and xylose, since the rate of reduction of DPN in the presence of glucose plus xylose is no greater than that in the presence of xylose alone. With the lens extract used in this experiment, the difference between glucose and xylose as substrates was not so great as with the extracts used in Fig. 1.

The oxidation of xylose by DPN was carried out on a larger scale in order to compare the product of oxidation with the xylonic acid formed on incubation of whole lenses in a xylose-containing medium. A dialysed lens dispersion was brought to pH 8·2 by careful addition of 0·1 N-KOH. A volume (1 ml.) of this dispersion, DPN (1 μmole) and xylose (67 μmoles), in a final volume of 3 ml., were incubated for 4 hr. at 37°. A blank was run without DPN. The reaction was stopped by adding 6 ml. of 7·5% (w/v) trichloroacetic acid to each tube, and the mixtures were centrifuged. The extracts were shaken three times with ether, neutralized, concentrated and taken up in 0·3 ml. of water. Chromatography

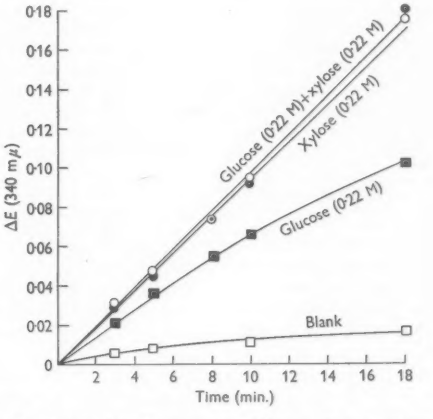

Fig. 2. Xylose dehydrogenase in calf lens; glucose, xylose and glucose plus xylose as substrates. Contents of Beckman cells were as described in Fig. 1, but with 660 μmoles of xylose or 660 μmoles of glucose, or 660 μmoles of each.

in phenol–water showed a large spot in the position of xylonic acid which appeared only in the extract from the incubation containing DPN.

Preparation of cadmium bromide–cadmium xylonate double salt from xylonic acid made from xylose by calf lens

The identity of xylonic acid formed from xylose by calf lens was confirmed by converting it into cadmium bromide–cadmium xylonate double salt, $(CdC_5H_9O_6)_2,CdBr_2,2H_2O$, which forms characteristic whetstone-shaped crystals (Elsner, 1935). It was not found possible to precipitate the crystals from xylonic acid in extracts of whole lens incubated in xylose-containing media, probably because of interfering compounds. The following method was therefore used.

Calf lens was ground with its own weight of water and dialysed against distilled water overnight at 4°. This dispersion (15 ml.) was cautiously brought to pH 8·2 with 0·1 N-KOH and incubated for 6 hr. with xylose (1000 μ-moles), sodium pyruvate (500 μmoles) and DPN (about 10 μmoles). The presence of pyruvate ensured the continual regeneration of the oxidized form of DPN, through lactic dehydrogenase. After 6 hr. the incubation mixture was diluted with an equal volume of water, brought to pH 5·5 with dil. acetic acid, placed in a boiling-water bath for 3 min. to precipitate protein, cooled and centrifuged. A chromatogram of 10 μl. of the clear extract in phenol–water showed that about 10% of the xylose had been oxidized to the acid. The rest of the extract (containing very approx. 100 μmoles of xylonic acid) was evaporated to dryness *in vacuo*. The residue was taken up in 0·5 ml. of water and centrifuged to remove remaining protein; 0·1 g. of $CdBr_2,4H_2O$ and 0·1 g. of $CdCO_3$ were then added to the extract, which after 30 min. at room temperature was evaporated almost to dryness on a water bath. Water (2 ml.) was added and the mixture stirred and centrifuged. The supernatant was reduced to 0·1 ml. on a water bath, cooled and 0·15 ml. of absolute ethanol added. After some days there was a heavy deposit of crystals in the extract, a large proportion of them being identical in appearance with those formed by the oxidation of xylose by bromine in the presence of cadmium carbonate (Elsner, 1935).

Chromatography of the fraction containing barium salts soluble in 80% (v/v) ethanol

This fraction was examined for the presence of xylulose, by chromatography and a combination of the trichloroacetic acid–orcinol and aniline phthalate sprays. Extracts of lens incubated in medium containing glucose plus xylose gave a small spot in the position of glucose and a large spot in the position of xylose, but nothing else. Cochromatography of these extracts together with authentic xylulose in a mixed spot showed that if 0·05% of the xylose present in the lens had been converted into xylulose its presence would have been detectable. The medium in which the lenses had been incubated was also examined for xylulose, but none was found. Comparisons of extracts from lenses incubated in medium with and without xylose confirmed the finding that less glucose is present in the lenses incubated with xylose in the medium although both media contained the same concentration of glucose.

Chromatography of fraction containing insoluble barium salts

The concentrated extracts were chromatographed in propan-1-ol–aq. NH_3 soln.–water, and the dried papers examined for nucleotides in ultraviolet light. Although the size of this fraction was much smaller in the lenses incubated in medium containing xylose (Table 7), the relative proportions of the four main nucleotides, ATP, ADP, UTP, UDP, appeared to be the same in the extracts from incubations in the presence or absence of xylose. This fraction was not examined further.

Effect of incubation with xylose on phosphorylcholine, glycerophosphorylcholine, phosphorylethanolamine and glycerophosphorylethanolamine in intact lenses

Chromatography of the fraction containing barium salts insoluble in 80% (v/v) ethanol (see earlier) showed no change in PC and GPC in lenses incubated with or without xylose. Precipitation of these compounds may not be quantitative under these conditions and a comparison was therefore made of extracts of type (iii) (see Methods), from lens incubated in media with or without xylose. These contain PC, GPC, PE and GPE (van Heyningen & Pirie, 1958). Five lenses were used in each group; the extract from the lenses incubated in glucose alone contained 513 μg. of P, and that from lenses incubated in glucose plus xylose 456 μg. of P. The difference is unlikely to be significant, considering the number of experimental manipulations involved.

Glutathione and ascorbic acid in the lens after incubation

Glutathione and ascorbic acid were measured in three pairs of lenses after incubation with and without xylose. The presence of xylose in the medium caused no change in the concentration of either of the substances.

DISCUSSION

The addition of xylose to the glucose-containing medium in which intact calf lenses were incubated brought about the following metabolic changes: (1) depression of formation of lactic acid (Table 4); (2) decrease in nucleotide di- and tri-phosphates (see Table 7); (3) increase in αGP; (4) formation of xylonic acid. These findings all fit in with the assumption that DPN, normally available for the

oxidation of 3-glyceraldehyde phosphate (GAP) to 1:3-diphosphoglyceric acid in the glycolytic pathway, is preferentially reduced by xylose dehydrogenase in the presence of xylose. The Michaelis constant for the use of DPN by glucose dehydrogenase in liver is 4·3 μM at pH 7 (Brink, 1953), whereas that for DPN in GAP dehydrogenase in muscle is 39 μM (Cori, Slein & Cori, 1948). Thus if the enzymes in lens are the same as these, the xylose dehydrogenase would be expected to have a greater affinity for DPN than that of the GAP dehydrogenase.

Reoxidation of reduced DPN, normally occurring through the action of lactic dehydrogenase on pyruvate, would in the presence of xylose be brought about instead by the action of αGP dehydrogenase on dihydroxyacetone phosphate. Owing to the blockage of GAP dehydrogenase the concentration of GAP, and of its isomeric form dihydroxyacetone phosphate, would be expected to be at an increased level. It is assumed that the rate of isomerization is not limiting.

$$\text{Xylose} + \text{DPN}^+ \rightarrow \text{xylonic acid} + \text{DPNH} + \text{H}^+ \quad (1)$$

$$\text{DPNH} + \text{dihydroxyacetone phosphate} \rightarrow \text{DPN} + \alpha\text{GP} \quad (2)$$

Sum: Xylose + dihydroxyacetone phosphate → xylonic acid + αGP

Thus the glycolytic cycle is interrupted, production of lactic acid is prevented, and ATP converted into ADP in the earlier unobstructed reactions of the glycolytic cycle is not able to be regenerated.

However, analysis of insoluble barium salts, containing nucleotide di- and tri-phosphates, did not show a higher proportion of ADP in lenses incubated in a xylose medium, although the whole fraction was considerably smaller (Table 7). Presumably other unidentified reactions are also involved.

The presence of an active glycerophosphate dehydrogenase in the calf lens is shown by the finding that DPN, reduced by a dialysed lens extract in the presence of xylose, is rapidly reoxidized on the addition of triose phosphate (Fig. 3).

Xylose interferes with the production of lactic acid from glucose by an intact lens (Table 4), but not by a lens dispersion (Table 1), although xylonic acid and glycerophosphate are formed in both cases. Presumably when the lens fibres are disrupted sufficient DPN is available both for reactions (1) and (2) and for the reactions in the glycolytic cycle. Even in the intact lens, the glycolytic cycle continues to operate to some extent, as shown by the formation of lactic acid, which is decreased but not abolished.

The xylose dehydrogenase in lens has not been examined in detail but it does not appear to be identical with the glucose dehydrogenase of liver (Strecker & Korkes, 1952; Brink, 1953). TPN, as well as DPN, is a coenzyme of the liver enzyme, but not of the lens enzyme. In the liver xylose is oxidized at a slower rate than glucose, whereas in the lens it is oxidized faster, especially at low concentrations of sugar. The possibility that D-xylono-γ-lactone is an intermediate in the formation of xylonic acid by the lens has not been investigated.

The findings presented in this paper accord with those of Wachtl & Kinsey (1957), who used rabbit lenses cultured *in vitro* under conditions which maintained a normal level of mitosis in the epithelial cells for 9 hr. They showed that substitution of glucose in the medium by xylose (both 1·25 mg./ml.) abolished the formation of lactic acid, although some xylose was consumed; lactic acid was formed when lenses were incubated with glucose and xylose together (each at a concentration of 0·625 mg./ml.), and some of each of these sugars was metabolized. Xylose alone did not support mitosis in the lens epithelium; when present together with glucose, the mitotic activity was about half that seen in the presence of glucose alone.

There are no figures in the literature for phosphorus in the lens in xylose cataract. In galactose cataract in rats, and also in diabetic cataract induced with alloxan, it has been shown that, when the lenses are partially opaque, compounds containing acid-labile P are decreased in concentration and P_i is unchanged (Nordmann & Mandel, 1953). Müller *et al.* (1955) used paper electrophoresis to

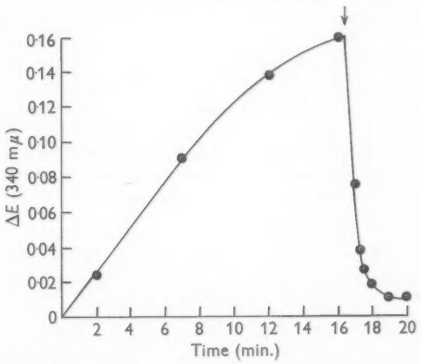

Fig. 3. Reduction of DPN by xylose dehydrogenase and reoxidation by glycerophosphate dehydrogenase. Beckman cell contained 1·5 ml. of dialysed lens extract, 1·4 ml. of phosphate buffer (M, pH 8) containing xylose (67 μmoles) and DPN (approx. 0·02 μmole). Triose phosphate (1 μmole of dihydroxyacetone phosphate and 1 μmole of glyceraldehyde phosphate) was added where indicated by the arrow.

compare the lenses of rabbits made diabetic with alloxan with normal rabbit lenses after incubation in a medium containing ^{32}P. They found that the lenses from the diabetic rabbits incorporated a smaller proportion of ^{32}P into ATP, and a correspondingly larger proportion into a fraction consisting chiefly of phosphorus compounds not hydrolysed in 7 min. in N-HCl at 100°.

SUMMARY

1. Xylose is not phosphorylated by the calf lens; it does not interfere with the phosphorylation of glucose by lens dispersions.

2. The formation of lactic acid from glucose is inhibited by xylose in the intact lens but not in lens dispersions. Penetration of glucose into the intact lens is inhibited by xylose.

3. Xylose is oxidized by the calf lens (intact or dispersed) to xylonic acid by means of a dehydrogenase using diphosphopyridine nucleotide (DPN). Reduction of DPN by dialysed lens extract at pH 8 is faster with xylose (0·02 M) as substrate than with glucose at the same concentration.

4. Comparison between intact lenses incubated in a medium containing glucose, without or with the addition of xylose, shows that the formation of xylonic acid from xylose is accompanied by an increase in the amount of α-glycerophosphate (αGP) in the lens and a corresponding decrease in the acid-labile phosphate contained in the nucleotide di- and tri-phosphates. There is no change in orthophosphate or in the sum of phosphorylcholine, phosphorylethanolamine, glycerophosphorylcholine and glycerophosphorylethanolamine.

5. It is suggested that, in the presence of xylose, DPN normally available for oxidation of 3-glyceraldehyde phosphate in the glycolytic cycle is preferentially reduced by xylose dehydrogenase to xylonic acid. The observed increase in αGP is explained on the assumption that the reduced DPN thus formed is reoxidized, not as in the normal lens through pyruvic acid and lactic dehydrogenase, but by the action of αGP dehydrogenase on dihydroxyacetone phosphate.

I wish to record my thanks to Dr A. Pirie and Dr S. G. Waley for helpful advice and encouragement; to Professor F. Dickens for a useful discussion; to the National Council to Combat Blindness Inc., New York, for FIGHT FOR SIGHT grants for the purchase of equipment; to Miss Joyce Blackwell and Mrs Eileen Davies for the collection of calves' eyes; and to the staffs of the Eastwyck Farm and Oxford Co-operative Slaughterhouses for their help.

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The Heterogeneity of β_2-Lactoglobulin

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The discovery by Aschaffenburg & Drewry (1955, 1957) that individual cows differed in the β-lactoglobulins that they produce (they have either β_1- or β_2-lactoglobulin or a mixture of the two), although going far to explain the heterogeneity of β-lactoglobulin prepared indiscriminately from mixed milk, does not completely account for all the observed indications of heterogeneity.

Thus Ogston & Tombs (1957) found that both β_1- and β_2-lactoglobulin gave complex boundaries on electrophoresis, and although most of this heterogeneity could be explained as due to polymerization in solution (Ogston & Tilley, 1955) there remained a possibility that some of the electrophoretic heterogeneity was due to the presence of distinct minor components.

This possibility was investigated for β_1-lactoglobulin mainly by use of solubility tests (Tombs, 1957), and the presence of about 10% of a minor component was detected. It could be removed by salt fractionation; the purified β_1-lactoglobulin had a constant-solvent solubility curve, and a variable-solvent solubility curve characteristic of a very nearly homogeneous substance. It still had a complex boundary in electrophoresis, but this could be completely explained as due to polymerization in solution. Some results obtained with β_2-lactoglobulin by using an approach similar to that already described for β_1-lactoglobulin are reported here.

EXPERIMENTAL

Experimental details of the preparation of β-lactoglobulins, electrophoresis, determination of concentration and measurements of ultraviolet absorption were the same as in Ogston & Tombs (1957). Solubility tests were substantially the same as were used for β_1-lactoglobulin (Tombs, 1957).

Variable-solvent solubility curves

The protein was dissolved in 1·6M-sodium chloride and dialysed against it for at least 24 hr. This solution was then mixed with an equal volume of acetate buffer (pH 4·5; 1·6M-sodium acetate, 2·9M-acetic acid) and samples (1 ml.) were placed in centrifuge tubes. Then 3 ml. of an appropriate ammonium sulphate solution was run in to give final concentrations of 0·2M-sodium chloride, 0·2M-sodium acetate and ammonium sulphate in the range 1·9–2·4M.

After initial experiments in which equilibration took place at 25° (Fig. 2) it was found that cooling was necessary in order to obtain amorphous precipitates; subsequently therefore all solutions were precooled to 4° before mixing, and the tubes were kept in a water bath at 4° during equilibration. The tubes were sealed with silicone-treated rubber bungs. After centrifuging at 4° (10 000 g for 20 min.), 2 ml. of the supernatant was removed from each tube and diluted to 3 ml. with water. β_2-Lactoglobulin has a negative temperature coefficient of solubility in these solvents and dilution was necessary to prevent precipitation occurring when the tubes warmed up to room temperature. Protein concentration in the diluted supernatants was estimated directly by the absorption at 278 mμ in 1 cm. cells.

Constant-solvent solubility curves

β_2-Lactoglobulin was dissolved in solvent containing 0·8M-sodium chloride, 0·8M-sodium acetate, 1·35M-acetic acid (pH 4·5), and dialysed against the same solvent. Then volumes of the β_2-lactoglobulin solution not exceeding 1 ml. were carefully measured into centrifuge tubes with an Agla micrometer syringe. Then each sample was made up to 1 ml. with diffusate, and 3 ml. of ammonium sulphate solution was added to give the desired final concentration. The volume of protein solution added to each tube gave a measure of the total amount of protein in the system, and the amount in solution was found by measurement of the absorption of the supernatants at 278 mμ. Tubes were equilibrated at 4°, and supernatants diluted after centrifuging in exactly the same way as for the variable-solvent test.

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Fractionation experiments

About 2 g. of β_2-lactoglobulin was dissolved in 20 ml. of solvent (0·8 M-sodium chloride, 0·8 M-sodium acetate, 1·35 M-acetic acid; pH 4·5) and 60 ml. of ammonium sulphate solution added to give a final concentration of 2·15 M-ammonium sulphate. The mixture was equilibrated at 4° and then the precipitated protein was collected by centrifuging in the no. 30 head of a Spinco model L preparative ultracentrifuge at 29 000 rev./min. for 45 min. (about 50 000 *g*). The supernatant was then saturated with ammonium sulphate and the precipitated protein was obtained by centrifuging as before. The precipitates were dissolved in the appropriate solvents for further tests.

RESULTS

Electrophoresis

The behaviour of β_2-lactoglobulin in acetate buffers at pH 4·66 has already been reported (Ogston & Tombs, 1957). Resolution was poor, and a pH value was sought at which resolution might be better. Fig. 1 shows typical boundaries. All the runs were in acetate buffers (0·1 M-sodium acetate, and then acetic acid was added to give the required pH) between pH 4·66 and 5·58. Resolution appeared to be best at pH 5. β_1-Lactoglobulin gave very similar results in the same buffers.

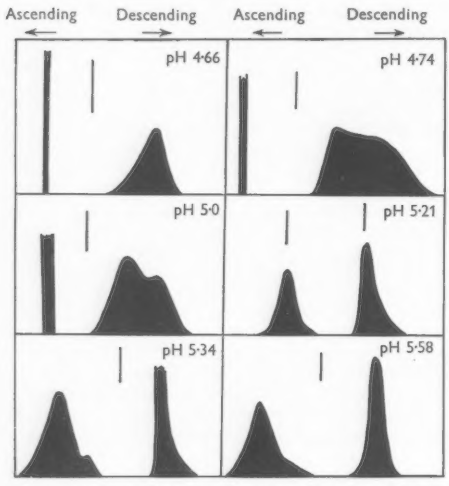

Fig. 1. Free electrophoresis of β_2-lactoglobulin. The buffer throughout was 0·1 M-sodium acetate and then acetic acid was added to give the required pH. Approximate starting positions are indicated by the vertical lines. All measurements were made after about 150 min. Two starting lines are shown at pH 5·21 for convenience of drawing.

pH	Protein concn. (g./100 ml.)	Current (mA)
4·66	0·96	10
4·74	3·0	10
5·0	1·1	10
5·21	1·25	20
5·34	1·25	20
5·58	0·98	10

Solubility experiments. Equilibration at 25° gave solubility curves which appeared satisfactory (Fig. 2), but on examination the precipitates were found to be crystalline. Very fine needle-like crystals were found in the tubes corresponding to part *A* of the curve, and small hexagonal crystals, together with amorphous material predominated in part *B*. The initial precipitates were always amorphous but at pH values 4·95, 4·8 and 4·5 at room temperature (15–17°) or at 25° the initial precipitates became partially or wholly crystalline in less than an hour. The crystals sometimes appeared in sheaves similar to those described by Smithies (1954). After 24 hr. the curves had the characteristic sigmoid shape described by Smithies, and the precipitates were completely crystalline. Such curves were useless for analytical purposes.

When the solubility tests were performed at 4°, no crystallinity was ever seen in the precipitates, even after equilibration for several days, on examination at × 600.

β_2-Lactoglobulin had a negative temperature coefficient of solubility in these solvents, and precipitate appeared when the ultraviolet absorption of the supernatants was measured at room temperature. This difficulty was overcome by diluting the supernatants (see Experimental section), but it was also possible to obtain reproducible curves by allowing the tubes to warm up to room temperature and centrifuging within an hour. Re-equilibration to room temperature appeared to

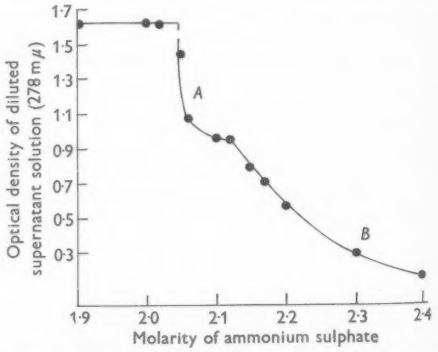

Fig. 2. Variable-solvent solubility curve of β_2-lactoglobulin equilibrated for 6 hr. at 25°, pH 4·5. Needle crystals appeared at *A*, hexagons at *B*. Details are given in the Experimental section.

be complete, no crystals could be seen and curves like those in Fig. 3 were obtained at several different protein concentrations, showing the point *T* to be invariant. The dilution technique was thought to be more reliable, however.

Curves obtained at two different protein concentrations are shown in Fig. 3. Several breaks appeared, of which *T* and *Y* appeared to be nearly invariant, though the total solubility at *Y* was higher at the higher protein concentration. The break at *X* could be distinguished only at higher protein concentrations. That at *Z* also appeared to move along the salt axis when protein concentration was changed. These breaks, especially at *X* and *Y*, were never very clearly defined.

A suitable solvent for the constant-solvent test was selected from a consideration of the variable-solvent solubility curve. The plot at 2·12M-ammonium sulphate is shown in Fig. 4. It indicated the formation of a solid solution, and no sharp breaks could be distinguished. At a higher ammonium sulphate concentration (2·17M) a precipitate again appeared well before maximum solubility had been reached. It was not possible to determine whether there were any sharp breaks or not.

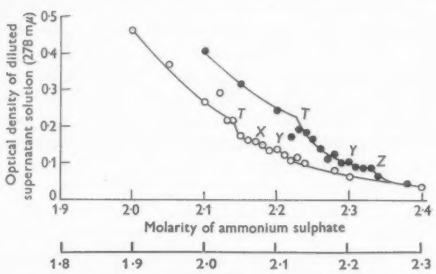

Fig. 3. Variable-solvent solubility curve of β_2-lactoglobulin equilibrated at 4° for 24 hr., pH 4·5. Values on the upper scale of the abscissa refer to the curve at the left (○), those on the lower scale of the abscissa refer to the curve at the right (●). Total protein concentrations (○, ●) were in the ratio 2:1. For *T*, *X*, *Y* and *Z* and experimental details see text.

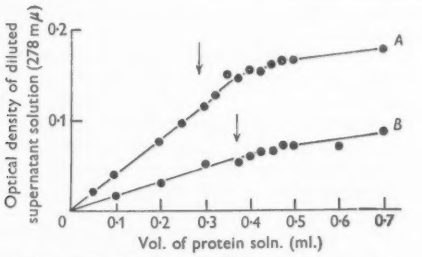

Fig. 4. Constant-solvent solubility curves of β_2-lactoglobulin. The solvent for *A* was 2·12M-ammonium sulphate, for *B* 2·17M-ammonium sulphate and in each case the solvent also contained 0·2M-sodium chloride, 0·2M-sodium acetate (pH 4·5). Equilibration was for 24 hr. at 4°. Precipitate first appeared at the points indicated by the arrows.

Fractionation experiments

The precipitate protein and the supernatant protein produced by fractionation with 2·15M-ammonium sulphate were examined by free electrophoresis. At pH 4·66 in acetate buffer ($I = 0·1$) at 1° a 2% solution of precipitate protein had a very sharp ascending boundary and a partially resolved descending boundary with 57% of slow component by area analysis. Unfractionated protein under the same conditions had 56% of slow component. A solution of supernatant protein (0·96%) had a skew-descending peak suggesting slightly more fast component than in the unfractionated protein at the same concentration. Resolution was poor at pH 4·66, and acetate buffer ($I = 0·1$) pH 5 was substituted. Supernatant protein, precipitated protein and unfractionated β_2-lactoglobulin were all run in the same batch of buffer, all at 1·5% of total protein. Area analysis of the descending boundaries gave 53% of slow component in the supernatant protein, 57% in the unfractionated and 60% of slow component in the precipitate protein. This difference, although small, was outside the usual error (±1%) for photographs of comparable resolution, and is probably significant.

The precipitate protein was also examined by the constant-solvent solubility test. The precipitate appeared before saturated solubility was reached and a plot made after equilibration for 24 hr. was characteristic of a solid solution. Equilibration for 3 days gave an almost identical plot, and the combined results of both experiments are shown in Fig. 5.

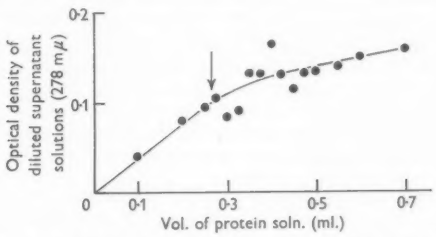

Fig. 5. Constant-solvent solubility curve of the 'precipitate protein' from β_2-lactoglobulin fractionation. The solvent was as for Fig. 4 (*A*). Curve shows the combined results of two experiments in which equilibration took place at 4° for 1 and 3 days. Precipitate first appeared at the point indicated by the arrow.

DISCUSSION

Because of their lower precision (two extra pipetting stages were introduced) and their more complex nature, the variable-solvent solubility curves of β_2-lactoglobulin were more difficult to interpret than those of β_1-lactoglobulin. Nevertheless, there are some points of similarity. The invariant point T in Fig. 3, and almost certainly Y also, were transition points between the precipitation of one solid form and another. Again, as with β_1-lactoglobulin, breaks like X, and possibly Z also, indicated the presence of minor distinct components. If the specific extinction coefficients of all the components were similar, the minor components formed rather less than 10% of the total.

The constant-solvent solubility curves, unlike those obtained with β_1-lactoglobulin, indicated formation of solid solutions. The solids might have been formed from one component, or they might have been solid solutions of one of the minor components in one or more solids formed by the major component. These solid solutions appeared to be stable and, since it was not possible to distinguish the various possibilities, the constant-solvent solubility curves did not provide clear evidence for or against heterogeneity.

The fractionation experiment, which was designed to leave the suspected minor components in the supernatant, and give a purified major component as the precipitate, supports the variable-solvent solubility experiments in indicating a small degree of heterogeneity. The constant-solvent solubility curve obtained with the precipitate protein emphasizes the ambiguity of this test in this particular set of circumstances.

The crystallization of β_2-lactoglobulin under conditions where β_1-lactoglobulin does not and apparently will not crystallize is a minor though striking difference between the proteins, and the fact that different kinds of crystal appear in parts of the solubility curve roughly corresponding to the precipitation of different amorphous solids suggest that the latter may be microcrystalline versions of the large crystals. The existence of transition points in the solubility curves of the β-lactoglobulins points towards a short-range structure of some kind in the solids.

The electrophoresis results reported here and by Ogston & Tombs (1957) make it clear that β_2-lactoglobulin, like β_1-lactoglobulin, associates in solution. Below the isoelectric point (pH 5·2) the polymer migrates most rapidly, above it the monomer form. The association process is probably the source of the sharpening of the descending boundary above the isoelectric point. The polymerization of β_1-lactoglobulin in solution was found to fit the requirements of Gilbert's (1955) theory fairly well (Tombs, 1957). This theory predicts that at high concentration of protein the proportion of slow component (monomer) from area analysis after electrophoresis will approach zero, provided that there are more than two monomer units in each polymer and that the rate of equilibration is infinitely rapid compared with the rate of resolution.

β_2-Lactoglobulin does not fit these requirements, since at pH 4·66 it reaches a limiting proportion of 60% of slow component at high protein concentration. The minor components found could not account for even part of the excess of slow component, since they appear to travel as part of the fast boundary in electrophoresis.

It appears that in its association–dissociation behaviour β_2-lactoglobulin is more complex than β_1-lactoglobulin. Similarly, it was not possible to gain as clear an understanding of its solubility behaviour, and this again was more complex than that of β_1-lactoglobulin. On the whole, however, β_2-lactoglobulin appears to contain rather less minor contaminants than β_1-lactoglobulin. This may be useful since cows producing only β_2-lactoglobulin are much commoner than those producing only β_1-lactoglobulin.

SUMMARY

1. In acetate buffers below pH 5·2 β_2-lactoglobulin forms a partially resolved descending and a very sharp ascending boundary in electrophoresis. Above pH 5·2 the descending boundary is sharpened and the ascending boundary spread.

2. Application of the variable-solvent solubility test showed two transition points, and two breaks probably due to the presence of distinct minor components, in the solubility curve. The minor components probably did not exceed 10% of the total.

3. The constant-solvent solubility test gave results characteristic of formation of solid solution.

4. Fractionation designed to remove the minor components gave a major fraction with more slow component, and a fraction in which the minor components had been concentrated, with less slow component than the unfractionated protein.

5. The purified β_2-lactoglobulin gave a constant-solvent solubility curve, characteristic of a solid solution.

6. It is concluded that the major part of the electrophoretic heterogeneity of β_2-lactoglobulin is due to association in solution, even though theoretical predictions of its extent are not correct.

I should like to thank Dr A. G. Ogston, F.R.S., for his constant interest in this work. The investigation was supported by a grant from the Agricultural Research Council.

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Glycolysis by an Extract from Pea Seeds

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It appears that no complete well-characterized glycolytic system in extracts from plant tissues, capable of transforming hexoses and hexose monophosphates into pyruvic acid or carbon dioxide and ethanol, has been described.

The conversion of carbohydrate into ethanol (Beevers & Gibbs, 1954; James & Ritchie, 1955) and lactate (Barker & el Saifi, 1952) in intact plant tissues provided evidence for a functional glycolytic system. This has been supported by the identification of individual glycolytic enzymes and certain intermediates in extracts from plants (Axelrod & Beevers, 1956). More integrated systems were found by Tanko (1936) and Hanes (1940), who prepared pea-seed extracts capable of converting starch into hexose monophosphates and fructose 1:6-diphosphate (FDP). James, James & Bunting (1941) showed that the production of pyruvic acid by crude barley-leaf saps was increased by the addition of sugars, FDP and 3-phosphoglyceric acid (3-PGA). Stumpf (1950) obtained a pea-seed extract which formed carbon dioxide from FDP in the presence of arsenate by a series of reactions analogous to those of yeast and muscle glycolytic systems. However, phosphoglyceric kinase was absent and the pyruvic kinase yielded adenosine diphosphate (ADP) rather than adenosine triphosphate (ATP).

During investigations on the synthesis of sucrose from glucose 1-phosphate (G 1-P) and fructose by pea-seed extracts, evidence was obtained for the operation of a glycolytic system (Turner, 1954, 1957; Turner & Mapson, 1958). The crude pea extract was partially purified by centrifuging at 20 000 *g*, treatment of the supernatant with ammonium sulphate to 80 % saturation, suspension of the precipitated protein in water and dialysis against phosphate buffer. With hexose and hexose phosphates as substrates and with a yeast nucleotide fraction and Mg^{2+} ions as cofactors, the partially purified pea extract esterified inorganic phosphate and formed FDP and carbon dioxide. For sucrose synthesis the yeast nucleotide fraction could be replaced by a mixture of diphosphopyridine nucleotide (DPN), ATP and uridine monophosphate.

In the present investigation these observations have been extended. The pea extract was capable of quantitatively converting starch, hexoses and hexose phosphates into carbon dioxide and ethanol. The results indicated that a complete glycolytic system of the Embden–Meyerhof–Parnas type was responsible for these transformations. There was no evidence for the operation of alternate pathways of hexose phosphate degradation in the pea-seed extract.

EXPERIMENTAL

Preparation of enzyme extracts. The crude and partially purified pea extracts were prepared as described previously (Turner, 1957) except that the partially purified extract was dialysed against phosphate buffer at pH 6·6 instead of pH 7. The partially purified extract, which contained approx. 45 mg. of protein/ml., showed no decrease in glycolytic activity after 2 weeks at −15°.

Substrates and cofactors. Commercial preparations of glucose and fructose, which gave only one spot when examined on paper chromatograms with butan-1-ol–acetic acid–water (4:1:5, by vol.; Partridge, 1948) as solvent, were used.

ATP, ADP and DPN were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A., and glucose 6-phosphate (G 6-P) and 3-PGA from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

G 1-P was prepared by an unpublished method of Professor C. S. Hanes and Dr R. Hill. Fructose 6-phosphate (F 6-P) (Neuberg, Lustig & Rothenberg, 1943) and FDP (Neuberg & Lustig, 1942) were also prepared in the laboratory.

G 1-P was standardized by measuring the inorganic phosphate released in 7 min. in N-HCl at 100°. F 6-P, G 6-P, FDP and 3-PGA each moved as single phosphoric ester spots on paper chromatograms developed in propan-1-ol–water–aq. NH_3 soln. (6:1:3, by vol.; Hanes & Isherwood, 1949), and were standardized by estimating total organic phosphate. Confirmatory estimations of the fructose component of FDP and F 6-P by the method of Roe & Papadodopoulos (1954) agreed to within 5%.

The yeast nucleotide fraction was prepared as described previously (Turner, 1954, 1957).

Analytical methods

Identification and estimation of sugars. Glucose and fructose were separated by chromatography with either propan-1-ol–water–aq. NH_3 soln. (16:3:1, by vol.; Turner, 1953) or ethyl acetate–propan-1-ol–water (57:32:13, by vol.; H. G. Wager, unpublished work) as solvent. The individual sugars were detected and estimated by methods previously described (Turner, Turner & Lee, 1957).

Estimation of phosphoric compounds. Inorganic orthophosphate was determined by the method of Allen (1940) with the modification of Turner (1957). Phosphoric esters were separated on paper chromatograms (Hanes & Isherwood, 1949) and detected by the method of Wade & Morgan (1953). FDP was estimated by cutting out the area of the spot from the chromatograms and determining the total phosphorus content by the method of Allen (1940). 3-PGA was determined as described by Umbreit, Burris & Stauffer, (1951).

Aldolase and triose phosphate isomerase activity. Aldolase activity was demonstrated by the method of Stumpf (1948). Triose phosphate isomerase activity was detected by measuring the ratio of the individual triose phosphates formed from FDP (Stumpf, 1948).

Determination of ethanol. Ethanol was distilled from the enzymic digests and estimated by the method of Peel (1951).

Enzyme digests

Enzyme studies were carried out at 30° under anaerobic conditions with Warburg manometers gassed with commercial O_2-free N_2. The rate of either hexose utilization or CO_2 formation was used as a measure of glycolytic activity. A correction factor for CO_2 retention in the liquid phase (pH 6·5) was applied (Umbreit *et al.* 1951). Toluene (0·05 ml.) was added to the enzymic digests as a standard procedure. Digest samples were inactivated by the addition of trichloroacetic acid.

RESULTS

Preliminary observations indicated that the crude pea extracts possessed glycolytic activity. Glucose and fructose were utilized with the simultaneous esterification of inorganic phosphate and production of CO_2. The decrease in hexoses was accompanied by increases in hexose monophosphates and FDP, indicating that mechanisms for the formation of ATP were operating. The partially purified pea extract, which was free of glycolytic substrates and cofactors, was used for the more detailed study.

Cofactor requirement of the partially purified extract. The partially purified extract, supplemented only with phosphate buffer, did not utilize hexoses or hexose phosphates. The capacity of the system to convert these substrates into CO_2 was restored by the addition of Mg^{2+} ions and the yeast nucleotide fraction (Fig. 1).

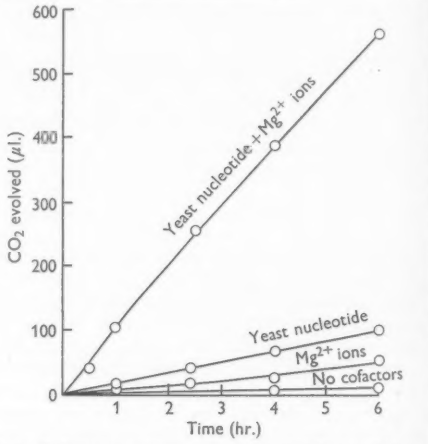

Fig. 1. Cofactor requirements of the partially purified pea extract. The complete system contained: enzyme, 1 ml.; sodium phosphate–potassium phosphate buffer, 25 μmoles (pH 6·5); G 1-P, 22 μmoles; fructose, 44 μmoles; $MgCl_2$, 18 μmoles; yeast nucleotide, 3·5 mg. Total vol. 1·8 ml.

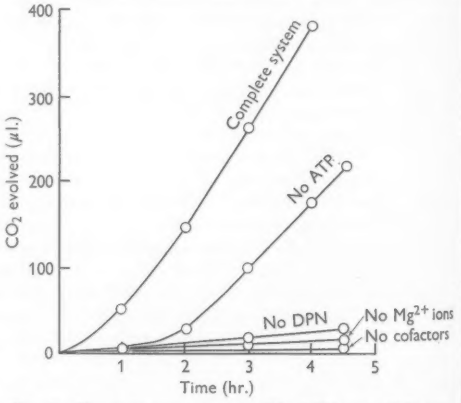

Fig. 2. The requirements for ATP, DPN and Mg^{2+} ions. The complete system contained: enzyme, 1 ml.; sodium phosphate–potassium phosphate buffer, 25 μmoles (pH 6·5); fructose, 44 μmoles; G 1-P, 22 μmoles; $MgCl_2$, 18 μmoles; ATP, 0·012 mM; DPN, 0·012 mM. Total vol. 1·8 ml.

A combination of ATP and DPN could replace the yeast nucleotide fraction (Fig. 2). In the absence of Mg^{2+} ions or DPN, glycolytic activity was negligible. The omission of ATP greatly increased the lag period and reduced the maximum activity by about 30%. Adenosine phosphates remaining after the purification procedure may have been sufficient to fulfil partially the requirements of the system. In some experiments the partially purified extract was dialysed against water instead of phosphate buffer, and inorganic phosphate was found to be essential for the utilization of hexoses and hexose phosphates. Arsenate could replace inorganic phosphate when FDP was used as the glycolytic substrate.

Production of carbon dioxide and ethanol from glycolytic substrates. Glucose, fructose, G 1-P, G 6-P, F 6-P, FDP, 3-PGA and starch (the extract contained starch phosphorylase) formed CO_2 when incubated with the partially purified extract and the appropriate cofactors (Table 1). As shown below, ethanol was also produced, the molar ratio ethanol:CO_2 being approx. 1.

With fructose or glucose alone as substrate, glycolytic activity was low (Table 1). The addition of a small amount of FDP initiated maximal steady-rate glycolysis of the hexoses, but the reasons for this are not apparent. The addition of fructose increased the rate of glycolysis of FDP to the level recorded for starch and hexose monophosphates. In separate experiments it was found that arsenate (5 mM) and ADP (10 mM) also increased the rate of FDP glycolysis to the same extent. The rate of CO_2 evolution from 3-PGA was considerably lower than the maximum observed rate with hexoses or hexose phosphates as substrates. This, as discussed subsequently, may have been due to an accumulation of acetaldehyde which inhibits pyruvic decarboxylase.

Hexose to fructose 1:6-*diphosphate stage*

When the phosphoric esters of samples taken from enzymic digests of the composition described in Table 1 were examined on paper chromatograms, it was observed that FDP was formed from hexoses or hexose monophosphates. When FDP was incubated with the extract, small amounts of hexose monophosphates and glucose were formed, indicating the presence of phosphatases acting on FDP and glucose monophosphates.

Fructose 1:6-*diphosphate to* 3-*phosphoglyceric acid stage.* With cyanide as a carbonyl fixative, evidence was obtained for the conversion of FDP into triose phosphate (Table 2). Estimation of the individual triose phosphates showed that dihydroxyacetone phosphate constituted over 95% of the triose

Table 1. *Breakdown of glycolytic substrates and intermediates to carbon dioxide*

Reaction mixtures contained: enzyme, 1 ml.; sodium phosphate–potassium phosphate buffer, 25 μmoles (pH 6·5); $MgCl_2$, 18 μmoles; ATP, DPN and substrates as indicated. Total vol. 1·8 ml. Glycolytic activity is expressed as an average of the first 4 hr. of steady-rate activity.

Expt. no.	Substrate	Amount of substrate (μmoles)	ATP (mM)	DPN (mM)	Glycolytic activity (μl. of CO_2/hr.)
1	None	—	1·5	0·35	0
	Starch*	66	1·5	0·35	110
	Glucose	44	1·5	0·35	18
	Glucose	44	0·75	0·35	96
	FDP	2·5	—	—	—
	Fructose	44	1·5	0·35	16
	Fructose	44	0·75	0·35	113
	FDP	2·5	—	—	—
	FDP	2·5	0·75	0·35	18
2	G 1-P	48	0·15	0·15	96
	G 6-P	40	0·15	0·15	92
	F 6-P	36	0·15	0·15	76
3	FDP	41	0·20	0·01	67
	FDP	62	0·20	0·01	64
	FDP	41	0·20	0·01	116
	Fructose	22	—	—	—
	3-PGA	62	0·20	0·01	72

* An additional 14 μmoles of sodium phosphate–potassium phosphate buffer was added in this experiment. Starch concentration is expressed as glucose equivalent.

Table 2. *Evidence for the glycolytic enzymes which convert fructose* 1:6-*diphosphate into* 3-*phosphoglyceric acid*

All reaction mixtures contained: enzyme, 1 ml.; sodium phosphate–potassium phosphate buffer, 25 μmoles (pH 6·5); FDP, 14·5 μmoles; $MgCl_2$, 18 μmoles; ATP, 0·2 mM; DPN, 0·2 mM; molybdate, 2·5 mM. In addition, the system which formed triose phosphate contained cyanide, 75 mM. The system which formed 3-PGA contained pyruvate, 130 μmoles; fluoride, 10 mM. Total vol. 2 ml.

Conversion of FDP into triose phosphate			Conversion of FDP into 3-PGA		
Time (min.)	FDP consumed (μmoles)	Triose phosphate produced (μmoles)	Time (min.)	FDP consumed (μmoles)	3-PGA produced (μmoles)
90	9·4	17·8	120	5·4	10·4

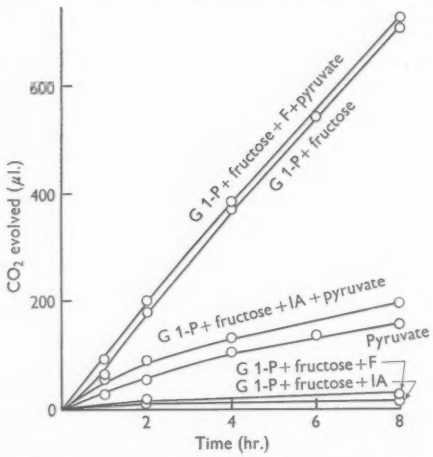

Fig. 3. Pyruvate decarboxylation and evidence for the DPN-linked coupling between triose phosphate dehydrogenase and alcohol dehydrogenase. All experiments contained: enzyme, 1 ml.; sodium phosphate–potassium phosphate buffer, 25 μmoles (pH 7); $MgCl_2$, 18 μmoles; yeast nucleotide, 3·5 mg. Additions, as shown, were: G 1-P, 22 μmoles; fructose, 44 μmoles; pyruvate, 108 μmoles; iodoacetate (IA), mM; fluoride (F), mM. Total vol. 1·8 ml.

phosphate formed. Both aldolase and triose phosphate isomerase were therefore functional.

In the presence of fluoride (to inhibit enolase activity) and pyruvate (to allow the DPN-linked coupling of triose phosphate dehydrogenase and alcohol dehydrogenase) the formation of 3-PGA from FDP was demonstrated (Table 2). The increase in organic phosphate resistant to hydrolysis in N-HCl at 100° for 3 hr. was attributed to 3-PGA, since molybdate was added to inhibit the phosphatase acting on FDP. Molybdate has previously been used as an inhibitor of certain plant phosphatases (Bailey, Thomas & Whelan, 1951; Turner & Turner, 1957).

Pyruvate to ethanol. The rate of pyruvate decarboxylation by the partially purified extract was relatively low (Fig. 3), and it was suspected that this was due to the accumulation of acetaldehyde. In the presence of a system producing reduced diphosphopyridine nucleotide (DPNH), which allowed the reduction of acetaldehyde to ethanol, the rate of pyruvate decarboxylation was increased four times (Fig. 3). DPNH was generated by the addition of fructose, G 1-P and fluoride; in the presence of fluoride, fructose and G 1-P were broken down to 3-PGA and DPNH was formed. The replacement of fluoride by iodoacetate, which inhibits triose phosphate dehydrogenase (Cori, Slein & Cori, 1948) and therefore DPNH production, eliminated the increased pyruvate decarboxylation.

When excess of fructose and G 1-P were added to a digest containing pyruvate and fluoride, a rapid rate of CO_2 production was maintained until the pyruvate was quantitatively decarboxylated. However, in the presence of fluoride and limiting amounts of fructose and G 1-P, the rate of CO_2 production from pyruvate declined markedly when the amount of CO_2 evolved was quantitatively equivalent to the complete conversion of the added fructose and G 1-P into 3-PGA.

Inhibitors of glycolysis. The addition of iodoacetate (mM) and fluoride (mM) resulted in an almost complete inhibition of CO_2 production from fructose plus G 1-P (Fig. 3). Similar results were obtained when G 1-P, F 6-P and FDP were supplied individually as substrates. Arsenate (10 mM) completely inhibited the glycolysis of fructose plus G 1-P but increased the rate of glycolysis of FDP.

Quantitative glycolysis of fructose, glucose 1-*phosphate and fructose* 1:6-*diphosphate.* The glycolysis of fructose, G 1-P and FDP produced ethanol and CO_2 in an approximately 1:1 ratio (Table 3). Approximately 93% of the added G 1-P was recovered as ethanol, CO_2 and inorganic phosphate, and 85–90% of fructose and FDP were accounted for as end products. The accumulation of inorganic

Table 3. *Quantitative glycolysis of fructose, glucose 1-phosphate and fructose 1:6-diphosphate to carbon dioxide and ethanol*

Reaction mixtures contained: enzyme, 1 ml.; sodium phosphate–potassium phosphate buffer, 25 μmoles (pH 6·5); $MgCl_2$, 18 μmoles; ATP, 0·35 mM; DPN, 0·35 mM; substrate as indicated. Total vol. 1·8 ml. Final measurements were made after 25 hr., when the rate of CO_2 production had fallen to zero in all experiments.

Substrate added (μmoles)		Product recovered (μmoles) CO_2	Ethanol	Inorganic phosphate
Fructose (21) + FDP (2·5)	Calc.	47	47	5·0
	Found	40	41	5·7
G 1-P (25·2)	Calc.	50·4	50·4	25·2
	Found	46	49	23·4
FDP (25)	Calc.	50	50	50
	Found	41	44	43·0

Table 4. *Adenosine triphosphate:carbon dioxide ratio*

Reaction mixture contained: enzyme, 1·2 ml.; sodium phosphate–potassium phosphate buffer, 29 μmoles; fructose 61 μmoles; FDP, 5 μmoles; $MgCl_2$, 18 μmoles; ATP, 0·36 mM; DPN, 0·018 mM. Total vol. 1·8 ml. The duration of the experiment was 5 hr.

Substance	Changes in digest (μmoles)	Calc. ATP consumption (μmoles)
CO_2 produced	15·2	—
Fructose consumed	18·0	18·0
FDP increase	0·6	0·6
FDP converted into CO_2 and ethanol	7·6	7·6
ATP production	Calc.* 30·4 ($ATP/CO_2 = 2$)	
	Found† 26·2 ($ATP/CO_2 = 1·73$)	

* Calculated from CO_2 production assuming classical glycolysis.

† Calculated from the experimental data assuming that ATP consumption is equal to ATP production.

phosphate rather than ATP was probably due to the action of phosphatases.

Reversal of glycolysis. An attempt to bring about a complete reversal of glycolysis by the addition of pyruvate, DPN, ethanol and substrate levels of ATP was unsuccessful. Neither hexose nor hexose monophosphates were formed in detectable amounts. However, incubation of pyruvate with substrate levels of ATP resulted in considerable formation of 3-PGA. Glucose was detected on paper chromatograms after the incubation of 3-PGA with DPN and substrate levels of ethanol and ATP. It was shown previously that glucose was formed from FDP in the presence of the pea extract.

Energetics of glycolysis: the adenosine triphosphate/carbon dioxide ratio. In the operation of the Embden–Meyerhof–Parnas glycolytic pathway two high-energy phosphate bonds should be generated for each molecule of CO_2 produced. The total ATP production was calculated from the experimental data assuming that ATP consumption was equal to ATP production. In calculating the ATP consumption it was assumed that the disappearance of one molecule of fructose and the appearance of one molecule of FDP, compared with initial levels, each required the utilization of one molecule of ATP. However, the net increase in FDP recorded did not represent the amount produced by the system during the course of the experiment. There was, in addition, the FDP which was formed and then converted into CO_2 and ethanol. It was assumed that one molecule of ATP was required for the formation of each molecule of FDP split in this manner. In Table 4 the calculated ATP production is compared with the theoretical production calculated from the CO_2 evolved. It was found that 87 % of the theoretical ATP production could be accounted for, giving an $ATP:CO_2$ ratio of 1·73.

DISCUSSION

The breakdown of starch, hexoses and hexose phosphates to carbon dioxide and ethanol by the partially purified pea extract was consistent with the operation of a glycolytic process. Additional evidence was provided by the conversion of hexoses and hexose monophosphates into FDP, FDP into triose phosphate and 3-PGA, and 3-PGA into carbon dioxide by the extract. The requirement for DPN, ATP and Mg^{2+} ions, and the inhibition by iodoacetate and fluoride, also indicated that glycolysis was operating.

Axelrod & Beevers (1956), in discussing possible alternative pathways of carbohydrate metabolism in plants, suggested that the pentose phosphate cycle could function anaerobically if reduced triphosphopyridine nucleotide generated by the system were oxidized. It has been found that certain intermediates of the pentose phosphate cycle may be converted into ethanol and carbon

dioxide *in vivo* under anaerobic conditions (Axelrod & Beevers, 1956). If the breakdown of hexoses and hexose phosphates by the pentose phosphate cycle occurred anaerobically, the carbon dioxide:ethanol ratio would be greater than 1. The recovery of 93–95% of G 1-P as carbon dioxide and ethanol in a 1:1 ratio indicated that mechanisms other than the Embden–Meyerhof–Parnas glycolytic pathway were not operating to an appreciable extent in the pea extract. The almost complete inhibition of glycolysis by low concentrations of iodoacetate and fluoride supported this conclusion. These observations do not exclude the possibility that additional mechanisms of hexose phosphate breakdown may operate in the intact pea seed.

There have been a number of reports of certain intermediary glycolytic conversions catalysed by cell-free plant extracts, and of these the system of Stumpf (1950) is of particular interest. Stumpf found that FDP was converted into carbon dioxide, provided that arsenate was added, but there was no evidence that ATP was produced. During glycolysis of hexoses and hexose monophosphates by the pea extract in the present investigation, the theoretical production of two molecules of ATP per molecule of carbon dioxide was approached. This indicated that both 3-phosphoglyceric acid kinase and pyruvic kinase produced high-energy phosphate bonds. The ATP formed was then available for the initial phosphorylations involved in the glycolysis of starch, hexoses and hexose monophosphates. These processes allowed the cyclic interconversion of ATP and ADP.

The addition of fructose increased the rate of glycolysis of FDP by the pea extract to the rate observed with starch or hexose monophosphates. This suggested that the level of ADP, which is required for the action of 3-phosphoglyceric acid kinase and pyruvic kinase, may have been limiting. This was supported by the observations that arsenate, which eliminates 3-phosphoglyceric acid kinase from the glycolytic sequence (Warburg & Christian, 1939), and ADP also increased the rate of FDP glycolysis.

There was some evidence that the low rate of production of carbon dioxide from pyruvate and 3-PGA by the pea extract was due to an accumulation of acetaldehyde. Acetaldehyde is known to inhibit pyruvic decarboxylase (Lohmann & Schuster, 1937). When enolase was inhibited by fluoride, the addition of fructose plus G 1-P resulted in an increase in the rate of pyruvate decarboxylation by the pea extract. It is probable that, under these conditions, alcohol dehydrogenase and triose phosphate dehydrogenase were coupled by DPN so that DPNH produced by the latter enzyme was available for acetaldehyde reduction.

SUMMARY

1. A partially purified extract from pea seeds, when supplemented with cofactor levels of adenosine triphosphate, diphosphopyridine nucleotide and Mg^{2+} ions, catalysed the conversion of starch, hexoses, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate into carbon dioxide and ethanol.

2. mM-Iodoacetate and -fluoride inhibited glycolysis by the pea extract.

3. Approximately 93% of glucose 1-phosphate and 85–90% of fructose and fructose 1:6-diphosphate were converted into carbon dioxide and ethanol. Within experimental error the ratio carbon dioxide:ethanol was 1.

4. The following intermediary glycolytic transformations were demonstrated: hexoses and hexose monophosphates to fructose 1:6-diphosphate; fructose 1:6-diphosphate to triose phosphate and to 3-phosphoglyceric acid; 3-phosphoglyceric acid and pyruvate to carbon dioxide.

5. Evidence was obtained for the operation of cycles involving the oxido-reduction of diphosphopyridine nucleotide and the interconversion of adenosine triphosphate and adenosine diphosphate.

6. The ratio of adenosine triphosphate formed to carbon dioxide produced was calculated from the experimental data. A value of 1·73, compared with a theoretical figure of 2, was obtained.

7. Reversibility of the glycolytic process was demonstrated in two stages. Pyruvate was converted into 3-phosphoglyceric acid and 3-phosphoglyceric acid into glucose.

8. The evidence indicated that a complete Embden–Meyerhof–Parnas glycolytic system was functioning. Alternative pathways of hexose degradation were not operating to a significant extent.

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The Metabolism of Adrenocorticotrophic Hormone and Ascorbic Acid in the Chick

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Depletion of adrenal ascorbic acid follows the administration of adrenocorticotrophic hormone in the rat or guinea pig but not in the chick (Jailer & Boaz, 1950), quail (Zarrow & Baldini, 1952) or duckling (Zarrow & Zarrow, 1950). These authors found that continued injections of adrenocorticotrophic hormone or a 'stress agent' such as stilboestrol did nevertheless produce adrenal hypertrophy in birds. They therefore concluded that the synthesis and release of adrenocortical hormones could occur without an effect on the adrenal ascorbic acid. This paper describes a further investigation of the influence of adrenocorticotrophic hormone and stress agents on ascorbic acid metabolism in the chick.

METHODS

Animals. Cockerels, 6–10 weeks old, obtained from local breeders and kept in wire cages in a chicken shed for about a week before experiment, were fed on a commercial chick crumb diet mixed with 5% of dried-grass meal. The birds used were Light Sussex × Rhode-Island Red, Light Sussex × North-Holland Blue and North-Holland Blue strains. In each individual experiment only one strain was used.

Male Wistar rats, laboratory bred, of 200–300 g. body wt. were used.

Male guinea pigs, of approx. 250 g. body wt., were obtained from Allington Farm, Ministry of Supply, Porton, Wilts, and before being placed on experiment were fed with a fish-meal diet (20% of protein) (Harris, Constable, Howard & Leader, 1956) supplemented by 50 mg. of ascorbic acid/guinea pig/day (orally). When the guinea pigs reached a body weight of 300 g., enough casein (Glaxo Laboratories Ltd., Greenford, Middlesex) was added to the diet to increase the protein content to 55%; the diet was damped immediately before use.

Adrenocorticotrophic hormone (ACTH). Lyophilized ACTH (Armour and Co. Ltd., Chicago, Ill., U.S.A.) was injected as a solution in saline (25 i.u./2·5 ml. of 0·9% sodium chloride soln.) when a single dose was given. Since a gelatin medium is said to enhance ACTH activity when given over long periods (Wolfson, 1953) a 16% gelatin solution of the hormone [25 i.u. dissolved in 0·5 ml. of saline, followed by addition of 2 ml. of 20% (w/v) gelatin] was used when several doses were to be administered.

Analytical methods. All animals were killed swiftly by dislocation of the cervical vertebrae. This was followed by decapitation and exsanguination. Whole blood from the jugular vein was collected carefully into a receptable containing a few crystals of sodium oxalate. The organs to be analysed were rapidly excised and estimations carried out immediately. Ascorbic acid was estimated in the adrenals and plasma by the method of Mindlin & Butler (1937) and in whole blood and plasma by that of Roe & Kuether (1943); adrenal cholesterol by the ferric chloride method of Zlatkis, Zak & Boyle (1953) as described by Knobil, Hagney, Wilder & Briggs (1954); liver glycogen by the method of Good, Kramer & Somogyi (1933) as modified by Somogyi (1937) and blood glucose as described by King (1951).

RESULTS

Effect of a single injection of adrenocorticotrophic hormone on adrenal ascorbic acid

ACTH (10 i.u.) injected intramuscularly into the rat produced, 1 hr. after injection, a 30% decrease in adrenal ascorbic acid concentration as compared with control animals injected with saline. In the chick, however, results were obtained similar to those of Jailer & Boaz (1950). Both intramuscular and intravenous injections had no effect 1, 2 or 6 hr. after injection (Table 1). After 6 hr., however, a 20% depletion of adrenal cholesterol occurred, a phenomenon which in the rat and guinea pig accompanies the fall in ascorbic acid produced by ACTH (Long, 1946).

Effect of adrenocorticotrophic hormone on carbohydrate metabolism

The deposition of liver glycogen which occurs in the adrenalectomized fasted rat or mouse after administration of adrenal hormones, is the basis of a method for their bioassay (Reinecke & Kendall, 1942; Venning, Kazmin & Bell, 1946). Blood-sugar levels are also elevated by the influence of these hormones. Since a primary effect of ACTH is to stimulate production of adrenocorticoids, it was of interest to determine whether these glycogenic properties could be induced by injections of ACTH in the fasted intact animal.

Cockerels were fasted for 24 hr. but allowed access to water. One group was then injected with five doses of ACTH (5 i.u. in 16% gelatin), at 1 hr. intervals, and killed 1 hr. after the last injection. A control group was injected likewise with 16% gelatin. On analysis, chicks receiving ACTH were found to have 20 times more liver glycogen and 25% more blood glucose than control animals (Table 2), although their adrenal ascorbic acid concentration was unchanged.

According to Olsen *et al.* (1944) the glycogenic response to adrenocorticoids in rats is increased by feeding a diet high in protein. In our preliminary experiments with guinea pigs the response to ACTH was also found to be enhanced by pre-feeding a high-protein diet. Guinea pigs were therefore fed with a 55% protein diet for 7 days before experiment, deprived of food for 24 hr. and then injected with five 1-hourly doses of ACTH (2 i.u. in 16% gelatin) as described above. Liver glycogen and blood glucose were found to be increased compared with controls injected with gelatin solution without ACTH (Table 2). In contrast with chicks, however, there was a concurrent fall in adrenal ascorbic acid concentration.

Table 1. *Effect of a single injection of adrenocorticotrophic hormone* (10 *i.u.*) *on adrenal ascorbic acid and cholesterol in the chick compared with the rat*

In this and in the other tables all results are given ± S.E. Values in which the difference from normal is statistically significant are denoted by asterisks.

Animal	Injection	Mode of injection	Time killed after injection (hr.)	No. of animals	Adrenal ascorbic acid (mg./100 g.)	Adrenal cholesterol (g./100 g.)	Blood ascorbic acid (mg./100 ml.)
Rat	Saline	Intramuscular	1	6	347 ± 23	—	—
	ACTH	Intramuscular	1	6	246 ± 20*	—	—
Chick	Saline	Intravenous	1	4	144 ± 5·9	—	—
	ACTH	Intravenous	1	4	136 ± 14·9	—	—
	Saline	Intravenous	6	4	138 ± 6·5	5·38 ± 0·13	1·16 ± 0·17
	ACTH	Intravenous	6	4	155 ± 4·3	3·85 ± 0·29**	1·18 ± 0·15
	Saline	Intramuscular	2	8	148 ± 5·1	—	—
	ACTH	Intramuscular	2	8	145 ± 6·8	—	—

* Denotes $P = 0{\cdot}05$–$0{\cdot}01$. ** Denotes $P = 0{\cdot}01$–$0{\cdot}001$.

Table 2. *Effects of five* 1*-hourly injections of adrenocorticotrophic hormone in fasted chicks and guinea pigs*

Each chick received a total of 25 i.u., and each guinea pig a total of 10 i.u. of ACTH.

Metabolite	Treatment	Chick†	Guinea pig‡
Liver glycogen (mg./100 g.)	Control	9 ± 4·1	102 ± 22
	ACTH	204 ± 45**	407 ± 114*
Blood glucose (mg./100 ml.)	Control	165 ± 10·8	99 ± 2·1
	ACTH	209 ± 6·0**	116 ± 3·5**
Adrenal ascorbic acid (mg./100 g.)	Control	176 ± 10·5	89·1 ± 3·9
	ACTH	172 ± 7·1	56 ± 6·6**

* Denotes $P = 0{\cdot}05$–$0{\cdot}01$. ** Denotes $P = 0{\cdot}01$–$0{\cdot}001$. † 8 animals/group. ‡ 6 animals/group.

Table 3. *Effects in chicks of continuous injections of adrenocorticotrophic hormone*

Chicks received 15 i.u. of ACTH/day for 8 days.

Treatment	No. of animals	Body wt. At start (g.)	Body wt. At end (g.)	Liver wt. (g.)	Adrenal wt. (mg.)	Adrenal cholesterol (g./100 g.)	Adrenal ascorbic acid (mg./100 g.)	Blood ascorbic acid (mg./100 ml.)
Control	6	891±22	1103±16	33·7±3·0	98±5·0	3·64±0·19	121±5·1	1·46±0·10
ACTH	6	887±19	951±18*	30·5±1·3	134±10·7**	1·69±0·10***	115±9·3	0·70±0·06***

* Denotes $P = 0{\cdot}05$–$0{\cdot}01$. ** Denotes $P = 0{\cdot}01$–$0{\cdot}001$. *** Denotes $P = <0{\cdot}001$.

Table 4. *Effect of pituitary stimulants on adrenal ascorbic acid in the rat and chick [with significance (P) between non-injected and injected animals]*

Animal	Injection	No. of animals	Adrenal ascorbic acid (mg./100 g.)
Rat	None	6	444±15
	Saline	6	348±23**
	Histamine	6	264± 7***
	Insulin	6	202±18***
Chick	None	7	115± 3
	Saline	6	121± 9
	Histamine	5	110± 3
	Insulin	6	111± 8

* Denotes $P = 0{\cdot}05$–$0{\cdot}01$.
** Denotes $P = 0{\cdot}01$–$0{\cdot}001$.
*** Denotes $P = <0{\cdot}001$.

Continued administration of adrenocorticotrophic hormone

Administration of ACTH to guinea pigs over a long period has been shown previously to result in enlargement of the adrenal gland, and a diminution in adrenal ascorbic acid concentration (Constable, Harris & Hughes, 1955). An increased liver weight also occurs in the guinea pig and rabbit, although not in the rat, chick or mouse (Harris, Bland, Hughes & Constable, 1953). In a continuation of these studies, cockerels were injected with ACTH (15 i.u. in 16 % gelatin) in divided doses twice daily for 8 days. A second group was similarly treated with gelatin solution without ACTH. As shown in Table 3, ACTH treatment caused a retardation in growth, an enlargement of the adrenals and a greatly diminished adrenal cholesterol concentration. Although there was no change in adrenal ascorbic acid concentration, an unexpected result was a 50 % diminution in the ascorbic acid content of the blood. A single injection of ACTH had no observable effect on blood ascorbic acid (Table 1). As noted previously, ACTH did not produce enlargement of the liver in the chick (Harris *et al.* 1953).

Pituitary stimulants and adrenal ascorbic acid

Injections of numerous substances are known to produce a fall in adrenal ascorbic acid in the intact rat. Since this effect is absent in the hypophysectomized animal, they are thought to act through the stimulation of the hypophysis and production of endogenous ACTH (Sayers & Sayers, 1948). In our experiments, groups of six rats were injected with saline at room temperature (0·05 ml./100 g. body wt.), histamine diphosphate (1·5 mg./100 g. in 0·05 ml. of saline) and insulin (2·5 i.u./100 g. in 0·05 ml.). In all instances the injected animals had a lower adrenal ascorbic acid concentration than non-injected controls (Table 4). The effect of histamine and of insulin was also greater than that of saline alone. No evidence was obtained that the above-mentioned phenomenon occurs in chicks, since injections of similar doses of these substances had no effect on their adrenal ascorbic acid (Table 4).

Sodium salicylate and ascorbic acid

The administration of salicylate also depletes the adrenal ascorbic acid of the rat, but it is thought that in this instance endogenous ACTH is not entirely responsible, since the effect occurs even in the hypophysectomized animal (Coste, Bourrel & Delbarre, 1953; Weidmann, 1955). In our experiments on the rat, an intramuscular injection of sodium salicylate (20 mg./100 g. in 0·1 ml. of saline) produced a marked decrease in adrenal ascorbic acid 2 hr. later (Table 5). With the same dose, however, no effect was observed in the chick. When a much larger dose (60 mg./100 g.) was administered to chicks, a small but significant decrease did occur. As shown in Table 5, with this dose there was also a rise in plasma ascorbic acid, as determined by two different methods.

DISCUSSION

The sharp fall of ascorbic acid in the rat or guinea-pig adrenal after the administration of ACTH (Sayers, Sayers, Lewis & Long, 1944) has been utilized for its assay. Although there is ample evidence that this effect is associated with an increased output of adrenal steroids (Bush, 1953), it appears to be confined to mammalian species. We have confirmed the observation of Jailer & Boaz (1950) that a single injection of ACTH is

Table 5. *Effect of injections of sodium salicylate on adrenal and plasma ascorbic acid in rats and chicks*

Plasma ascorbic acid was measured as described by Mindlin & Butler (1937) (*A*) and by Roe & Kuether (1943) (*B*).

Animal	Injection	No. of animals	Ascorbic acid: Adrenals (mg./100 g.)	Ascorbic acid: Plasma (*A*) (mg./100 ml.)	Ascorbic acid: Plasma (*B*) (mg./100 ml.)
Rat	Saline	6	348±10·1	—	—
	Salicylate (20 mg./100 g. body wt.)	6	228±10·7***	—	—
Chick	None	7	115± 3·0	—	—
	Salicylate (20 mg./100 g.)	6	111± 4·0	—	—
	Saline	9	121± 9·3	1·56±0·08	1·55±0·10
	Salicylate (60 mg./100 g.)	9	90± 6·3*	2·19±0·07***	2·06±0·07***

* Denotes $P = 0{\cdot}05$–$0{\cdot}01$. ** Denotes $P = 0{\cdot}01$–$0{\cdot}001$. *** Denotes $P = <0{\cdot}001$.

without effect on the adrenal ascorbic acid of chicks. We find that the depletion of adrenal cholesterol, which is a characteristic result of administration of ACTH to mammals, also occurs in the chick. Since adrenal cholesterol is considered to be a precursor of the adrenal corticoids, this finding suggests that they can in fact be synthesized in birds under the stimulation of the pituitary hormone. Further evidence was obtained by studying the effects of ACTH on carbohydrate metabolism. The administration of cortical hormones is known to produce an increase in liver glycogen and elevated blood-sugar levels in fasted chicks, as in other species (Golden & Long, 1942). Similar effects were observed in guinea pigs and chicks with ACTH. But whereas in guinea pigs a significant fall in adrenal ascorbic acid concentration was produced, this effect was absent in the chick. It can thus be inferred that ACTH is capable of stimulating the production of adrenal hormones in chicks without affecting adrenal ascorbic acid. It is unwise to conclude from these results that ascorbic acid is unnecessary for the synthesis of the adrenocorticoids, but the evidence is strongly suggestive. The elevated levels of adrenocorticoids found in the plasma (Done, Ely, Heiselt & Kelly, 1953) and in the urine (Burstein, Dorfman & Nadel, 1955) of scorbutic guinea pigs provide evidence to support this view.

As in other species, continued administration of ACTH to chicks resulted in an enlargement of the adrenals accompanied by a very low cholesterol content. This latter finding is usually associated with exhaustive stimulation of the adrenal (Sayers & Sayers, 1948). As in the rat (Asling, Reinhardt & Li, 1950), there is a retardation in growth. A surprising result was the greatly diminished concentration of ascorbic acid in the blood of chicks produced by prolonged injection of ACTH, an effect which was not apparent after a single injection. Further work is necessary before it can be established whether or not this effect is due to a direct interaction of ACTH and ascorbic acid. The concentration of ascorbic acid in the adrenals was unaffected.

Substances known to produce endogenous ACTH in the rat, such as histamine, insulin and saline, were without effect on adrenal ascorbic acid in the chick. In this respect our results agree with those of earlier workers who used adrenaline (Jailer & Boaz, 1950) and malarial infection (Taylor, 1952) as pituitary stimulants.

According to Cronheim, King & Hyder (1952) sodium salicylate (30 mg./100 g. body weight) acts as a pituitary stimulant in rats, and the ascorbic acid-depleting effect is prevented by hypophysectomy. On the other hand Coste *et al.* (1953), using a higher dose (50 mg./100 g.), found that salicylate is capable of eliciting a response even when the rat is hypophysectomized. Weidmann (1955) found that the depletion of adrenal ascorbic acid in hypophysectomized rats varied with the dose given. Coste *et al.* and Weidmann suggest that salicylate has a direct action on adrenal ascorbic acid. Our results agree with this view since we have found that in chicks a high dose of salicylate (60 mg./100 g.) was capable of depleting ascorbic acid, although a lower dose (30 mg./100 g.), which was active in normal rats, was ineffective. Moreover, the changes in ascorbic acid metabolism induced by high levels of salicylate were not confined to the adrenal since elevated plasma ascorbic acid was also observed. A rise in plasma ascorbic acid has also been noted in man dosed orally with salicylate (Stewart, Horn & Redson, 1953), and although the mechanism seems obscure the result is interesting in view of the chemical interaction *in vitro* of salicylates with ascorbic acid, involving hydroxylation of the aromatic nucleus (Brodie, Axelrod, Shore & Udenfriend, 1954).

SUMMARY

1. It has been confirmed that the transient fall in adrenal ascorbic acid after the administration of adrenocorticotrophic hormone (ACTH) in the rat does not occur in the chick.

2. A single injection of ACTH resulted in a depletion of adrenal cholesterol in chicks.

3. Evidence has been obtained that in the chick ACTH can stimulate the production of adrenal hormones affecting carbohydrate metabolism although having no effect on adrenal ascorbic acid.

4. The continued administration of ACTH to the chick also caused a retardation of growth, an enlargement of the adrenal and a decreased adrenal cholesterol concentration. Although the ascorbic acid was unaffected in the adrenal there was a fall in its concentration in the blood.

5. No evidence was obtained that endogenous ACTH produced by pituitary stimulants could deplete adrenal ascorbic acid in the chick.

6. Large doses of sodium salicylate caused the ascorbic acid to fall in rat and chicken adrenals and to rise in chicken plasma.

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Studies in the Biochemistry of Micro-organisms

105. CHEMICAL DEGRADATION OF SCLEROTIORIN*

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Sclerotiorin, a metabolic product originally obtained from the mycelium of *Penicillium sclerotiorum* by Curtin & Reilly (1940), was isolated by us (Birkinshaw, 1952) in considerably larger yield from *Penicillium multicolor*. The molecular formula put forward by Curtin & Reilly was corrected to $C_{21}H_{23}O_5Cl$ and the unsaturated acid obtained on alkaline hydrolysis was identified as (+)-4:6-dimethylocta-2:4-dienoic acid. This structure for the unsaturated acid was also arrived at independently by Watanabe (1952).

The work was continued and some products resulting from further degradation of sclerotiorin, and particularly of its compound with ammonia, indicating a possible partial structure, were mentioned in a communication (Birkinshaw, 1956) in

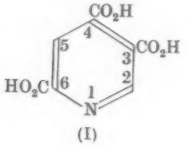
* Part 104: Raistrick & Stössl (1958).

which the antibiotic activity of sclerotiorin and of various breakdown products was the main consideration. As it came to our notice that a group of workers at Liverpool (Powell, Robertson & Whalley, 1956) were also investigating sclerotiorin, further work on its constitution was suspended, but it appears appropriate briefly to characterize the degradation products and to describe the method by which they were obtained. Some difficulty was experienced in obtaining crystalline breakdown products of sclerotiorin itself. The product obtained by the action of ammonia on sclerotiorin was examined and proved more amenable to chemical degradation. This compound ($C_{21}H_{24}O_4NCl$), already described (Birkinshaw, 1952) and now for convenience named sclerotiorazin, is readily reduced by zinc and acetic acid or by zinc and mineral acid in methanol to a yellow crystalline product sclerotazin ($C_{19}H_{22}O_2NCl$), m.p. 235–237° (decomp.), representing the addition of a molecule of hydrogen and loss of a molecule of acetic acid according to the equation

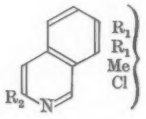
$$C_{21}H_{24}O_4NCl + H_2 \rightarrow C_{19}H_{22}O_2NCl + CH_3 \cdot CO_2H.$$

When hydrochloric acid or sulphuric acid was employed in the reduction the product was isolated as sclerotazin hydrochloride or sulphate respectively. Sclerotazin readily forms a crystalline diacetate, m.p. 150–151°, indicating that the two oxygen atoms are probably present as hydroxyl groups. When sclerotazin is subjected to very mild alkaline conditions a complex reaction seems to occur with formation of mainly amorphous products. One product was obtained in small yield in the form of orange plates, $C_{19}H_{23}O_3N$, m.p. 221–223° (decomp.), in which the chlorine atom of sclerotazin has apparently been replaced by a hydroxyl group.

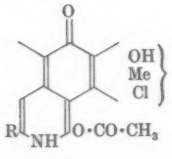
Ozonolysis converts sclerotazin diacetate into a crystalline diacetate $C_{15}H_{12}O_6NCl$, m.p. 235–236° (decomp.), which is acidic. This is evidently produced by removal of eight carbon atoms of the unsaturated side chain which gave rise to the dimethyloctadienoic acid produced by alkaline hydrolysis of sclerotiorin. Fission at the $\alpha\beta$ double bond has converted the α-carbon atom of this chain into a carboxyl group present in the new compound, named sclerazinic acid diacetate. The compound is deacetylated by boiling with dilute hydrochloric acid to sclerazinic acid, $C_{11}H_8O_4NCl,H_2O$, the additional molecule of water being presumably water of crystallization. Further evidence of the presence of a carboxyl group in sclerazinic acid was obtained by heating the acid in quinoline with copper chromite catalyst, when one molecular proportion of carbon dioxide was liberated. When sclerazinic acid is oxidized with alkaline permanganate, berberonic acid (I) and acetic acid are formed. In this oxidation three carbon atoms are eliminated in forming the larger fragment. It seems likely that two of these are originally present in a benzene ring fused with the pyridine nucleus at the positions indicated by the two adjacent carboxyl groups located at positions 3 and 4 in berberonic acid, the third carbon atom lost being present as a methyl group attached to this benzene ring and being eliminated in the acetic acid formed in the oxidation. The benzene ring must have as further substituents the two hydroxyl groups and the chlorine atom which have also been lost in the oxidation with permanganate. The penultimate stage in the degradation, sclerazinic acid, should therefore have the structure (II) derived from the diacetate (III). The side chain removed by ozonolysis can



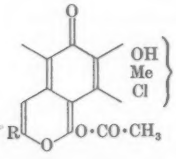
(I)



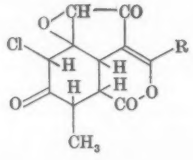
(II) $R_1 = \cdot OH$; $R_2 = \cdot CO_2H$
(III) $R_1 = CH_3 \cdot CO_2$; $R_2 = \cdot CO_2H$
(IV) $R_1 = CH_3 \cdot CO_2$; $R_2 = CH_3 \cdot CH_2 \cdot CHMe \cdot CH:CMe \cdot CH:CH \cdot$
(V) $R_1 = \cdot OH$; $R_2 = CH_3 \cdot CH_2 \cdot CHMe \cdot CH:CMe \cdot CH:CH \cdot$



(VI)



(VII)



(VIII)

In (VI), (VII) and (VIII), $R = CH_3 \cdot CH_2 \cdot CHMe \cdot CH:CMe \cdot CH:CH \cdot$

then be located at position 6 of the pyridine ring, adjacent to the nitrogen atom, where the lone carboxyl group stands in berberonic acid. Sclerotazin diacetate and sclerotazin are therefore accorded structures (IV) and (V) respectively.

The location of the carbon atoms liberated as acetic acid by mild reduction in the conversion of sclerotiorazin into sclerotazin is a more difficult problem. The evidence is inadequate to decide this point but a tentative structure (VI) was advanced as a possible solution of the structure of sclerotiorazin, leading to structure (VII) for the structure of sclerotiorin.

Compound (VI) represents a type of hemiquinonoid structure such as has been shown to exist in other fungal products, e.g. citrinin and fuscin. Reduction (the addition of 1 mol. of hydrogen) could be expected to transform it into an aromatic (*iso*quinoline) form with loss of acetic acid from the acetate grouping. Tautomeric forms would also be possible.

Methylamine reacts with sclerotiorin in a similar manner to ammonia, replacing one oxygen atom by :NMe to form *N*-methylsclerotiorazin, red crystals, m.p. 221°. It was earlier reported (Birkinshaw, 1956) that when this compound is reduced by zinc and acid it forms dihydro-*N*-methylsclerotiorazin by addition of two hydrogen atoms without loss of acetic acid. This statement, due to an erroneous analysis, is incorrect. It is now found that the reduction product (yellow needles, m.p. 239–241°), obtained by the action of zinc and acetic acid, has the molecular formula $C_{20}H_{26}O_3NCl$. Deacetylation has therefore occurred in addition to reduction, according to the equation

$$C_{22}H_{26}O_4NCl + H_2O + H_2 \rightarrow C_{20}H_{26}O_3NCl + CH_3 \cdot CO_2H.$$

The deacetylation has been confirmed by the detection of acetic acid as a product when the reduction is performed in methanol in the presence of zinc and sulphuric acid. The acetic acid appears in this case to arise by simple hydrolysis of an acetoxy group (replacement of acetoxyl by hydroxyl), not, as with sclerotiorazin itself, by complete elimination of a molecule of acetic acid (or hydrolysis followed by removal of water) leading to aromatization. This reaction is regarded as lending support to structure (VI) for sclerotiorazin. A structure (VIII) for sclerotiorin, somewhat similar to (VII), differing considerably, however, in the method of attachment of the two carbon atoms giving rise to acetic acid, was proposed by Powell *et al.* (1956).

EXPERIMENTAL

All melting points are uncorrected. Elementary analyses are by Weiler and Strauss, Oxford, or Imperial Chemical Industries Ltd., Akers Research Laboratories, Welwyn, Herts.

Light-absorption maxima of sclerotiorin and sclerotiorazin. Sclerotiorin: 227, 287, 362, 440–450 (inflexion) mμ in ethanol; log ε 4·20, 4·24, 4·60, 4·20 respectively. Sclerotiorazin: 235, 332, 500 mμ in ethanol; log ε 4·10, 4·68, 3·68 respectively.

Reduction of sclerotiorazin with zinc–acetic acid. Production of sclerotazin. Sclerotiorazin (1·0 g.) was stirred with Zn dust (1·0 g.) and acetic acid (50 ml.). The colour of the solution rapidly changed from intense red to yellow–orange. All the sclerotiorazin dissolved but some Zn remained and some zinc acetate crystallized out. When there was no further colour change (15 min.) the mixture was filtered and the residue on the filter was washed with acetic acid. The filtrate was poured into water (250 ml.) and the bright-yellow ppt. was allowed to settle, then collected and dried (wt. 1·0 g.). Recrystallization from methanol afforded golden-yellow prisms (double pyramids) of *sclerotazin*, m.p. 235–237° (decomp.). Light-absorption max. 223, 295, 410, 475 (inflexion) mμ in ethanol; log ε, 4·27, 4·61, 3·59, 2·59 respectively. The same product was obtained when propionic acid was substituted for acetic acid as solvent (Found: C, 68·9, 69·1, 68·95; H, 7·0, 6·8, 6·65; N, 4·2, 4·2, 4·35; Cl, 10·4, 10·7. $C_{19}H_{22}O_2NCl$ requires C, 68·8; H, 6·7; N, 4·2; Cl, 10·7).

Reduction of sclerotiorazin with zinc–sulphuric acid in methanol. Production of sclerotazin sulphate. Sclerotiorazin (1·0 g.) and Zn dust (1·0 g.) in methanol (50 ml.) were stirred and H_2SO_4 (50%, v/v; 1 ml.) was added to the mixture. The colour quickly changed through orange to golden-yellow (10 min.). The liquid was decanted into water (250 ml.), leaving the undissolved Zn. The bright-yellow flocculent ppt. was collected, washed with water and dried (wt. 0·97 g.). Recrystallization from methanol afforded lemon-yellow plates of *sclerotazin sulphate*, m.p. unsharp, mainly 174–178° after softening from 170° (Found: C, 57·3, 57·45; H, 6·3, 6·3; N, 3·7, 3·5; Cl, 10·2, 10·2; S, 4·2. $2C_{19}H_{22}O_2NCl,H_2SO_4,2H_2O$ requires C, 57·3; H, 6·3; N, 3·5; Cl, 8·9; S, 4·0%).

The aqueous filtrate from the sclerotazin sulphate was evaporated *in vacuo* almost to dryness and the evaporation was repeated twice after addition of water (50 ml. each time) to the residue. The distillates on titration were found to contain 18·77 + 0·43 + 0·11 ml. (total 19·3 ml.) of 0·1 N-volatile acid (75% of 1 mol. prop.). The neutralized distillates were concentrated to about 3 ml. and *p*-bromophenacyl bromide (0·54 g.) and ethanol (5 ml.) were added. The solution was refluxed for 1·5 hr. Addition of water to the warm solution to incipient turbidity produced crystals (0·43 g.) of m.p. 82–83°, raised on recrystallization to 85–86°. Admixture with authentic *p*-bromophenacyl acetate of the same m.p. produced no depression. Thus acetic acid is a product of the reaction.

Reduction of sclerotiorazin with zinc–hydrochloric acid in methanol. Formation of sclerotazin hydrochloride. The reaction was carried out as with H_2SO_4, with 2·0 ml. of conc. HCl in place of 1·0 ml. of 50% H_2SO_4. The product, a yellow powder, when crystallized from butan-1-ol, gave *sclerotazin hydrochloride* of m.p. 256–260° (decomp.) shrinking from 250° (Found: C, 61·4; H, 6·25; N, 3·5; Cl, 19·5. $C_{19}H_{22}O_2NCl,HCl$ requires C, 61·9; H, 6·3; N, 3·8; Cl, 19·35%).

Di-O-acetylsclerotazin. Sclerotazin (0·1 g.), anhydrous sodium acetate (0·2 g.) and acetic anhydride (1·0 ml.) were

heated together at 140° (bath temp.) for 30 min. The cooled mixture was poured into water and the cream-coloured solid (0·12 g.) so obtained was recrystallized from methanol, affording colourless needles of *sclerotazin diacetate*, m.p. 150–151°. The same product was obtained in similar yield by acetylation of sclerotazin sulphate by the same method (Found: C, 66·35, 66·5, 66·55; H, 6·4, 6·3, 6·3; N, 3·5, 3·5; Cl, 8·6, 8·7. $C_{22}H_{26}O_4NCl$ requires C, 66·4; H, 6·3; N, 3·4; Cl, 8·5%).

Mild alkaline hydrolysis of sclerotazin. Replacement of Cl *by* OH. (i) With NaOH. Sclerotazin (0·72 g.) was dissolved by warming in ethanol (60 ml.) and to the warm solution was added N-NaOH (2·2 ml., 1 equiv.). The solution, which rapidly darkened, was kept at room temp. for 18 hr. and then the ethanol was removed under reduced pressure. The residue was completely soluble in water. The intensely coloured aqueous solution was extracted with ether in a continuous extractor for 6 hr. On concentration of the ether solution to about 20 ml. a deep-red amorphous material (0·03 g.) separated and was removed. The ether was completely evaporated from the filtrate. The residue (0·39 g.) was crystallized from methanol, which afforded 70 mg. of orange plates with a bronzy lustre of *hydroxydechlorosclerotazin*, m.p. 221–223° (decomp.) (Found: C, 72·7; H, 6·8; N, 4·4; Cl, nil. $C_{19}H_{23}O_5N$ requires C, 72·8; H, 7·2; N, 4·5%). Further evaporation of the mother liquor afforded more orange plates and some double pyramids characteristic of sclerotazin. Only a small amount of amorphous ppt. (0·08 g.) was obtained from the ether-extracted aqueous solution on acidification.

(ii) With ethanolic potassium acetate. Sclerotazin sulphate (1 g.) dissolved in warm ethanol (150 ml.) was treated with potassium acetate in ethanol (50 ml. of 5%, w/v, solution). The mixture darkened and the deep-red solution gradually deposited colourless prisms (0·10 g.) of potassium chloride. After 10 days the solution was concentrated under reduced pressure to about 50 ml. and water (200 ml.) was added. Treatment of the emulsion obtained with 2N-H_2SO_4 (15 ml.) precipitated a dark-red solid (0·73 g.), from which by crystallization from methanol gleaming orange plates (0·05 g.) of m.p. 220° (decomp.) were obtained. This was the same product, hydroxydechlorosclerotazin, as that given by method (i) (Found: C, 72·7; H, 6·6; N, 4·5%; Cl, nil).

Ozonolysis of diacetate: sclerazinic acid diacetate. Sclerotazin diacetate (3·6 g.) dissolved in $CHCl_3$ (120 ml.) was treated with ozonized oxygen for 2·5 hr. The solution, yellow at first, slowly became almost colourless and towards the end of the period a small amount of flocculent ppt. appeared. The ozonized product was decomposed by shaking the solution with water (120 ml.) during 0·5 hr. An almost colourless solid (0·52 g.), m.p. 235–236° (decomp.) separated and was collected. The aqueous layer was discarded and the $CHCl_3$ layer was concentrated. Further fractions of the same solid were obtained (total wt. 1·88 g.). The final residue, an orange-red oil, was not further examined. Crystallization of the solid from ethyl malonate gave pale-cream microneedles of *sclerazinic acid diacetate* of m.p. 235–236° (decomp.) (Found: C, 53·25, 53·1; H, 3·75, 3·8; N, 4·0, 3·9; Cl, 10·5, 10·5. $C_{15}H_{12}O_6NCl$ requires C, 53·35; H, 3·6; N, 4·15; Cl, 10·5). The product is acidic since it dissolves in aqueous bicarbonate.

Sclerazinic acid. Sclerazinic acid diacetate (1·65 g.) was deacetylated by refluxing with N-HCl (400 ml.) until all the solid dissolved (1·5 hr.). The cooled solution was brought to pH 4·8–5·0 with 2N-NaOH. A lime-yellow ppt. slowly formed. The pptn. was completed at 0° and the product, *sclerazinic acid*, was collected (wt. 1·25 g.). It did not melt up to 360° but slowly darkened to a black pellet; light absorption max. 267, 354 mμ in ethanol; log ε 4·56, 3·62 respectively (Found: on material dried at 80°, C, 48·5, 48·7; H, 3·6, 3·75; N, 5·0, 5·0; Cl, 12·8, 13·0; loss in weight at 120°, 4·7. $C_{11}H_8O_4NCl,H_2O$ requires C, 48·6; H, 3·7; N, 5·2; Cl, 13·05; H_2O, 6·6%).

Sclerazinic acid hydrochloride. The diacetate (1 g.) was boiled under reflux with N-HCl (250 ml.) until all dissolved. Crystalline nodules separating were collected after 2 days and washed on the filter with dil. HCl. The product, *sclerazinic acid hydrochloride*, had m.p. not below 360°. The filtrate deposited a small amount of sclerazinic acid on adjusting to pH 5 (Found: C, 43·9; H, 4·5; N, 4·2; Cl, 20·1. $C_{11}H_8O_4NCl,HCl,H_2O$ requires C, 42·9; H, 3·6; N, 4·55; Cl, 23·0%). The substance has evidently lost some HCl on drying.

Decarboxylation of sclerazinic acid. Sclerazinic acid hydrochloride (0·504 g.), quinoline (7 ml.) and copper chromite catalyst (0·2 g.) were heated in a closed system with an air condenser. A current of CO_2-free N_2 was passed during the heating at bath temp. 160–170° for 3 hr. and at 220–230° for 3 hr. The issuing gases were bubbled through standard aq. $Ba(OH)_2$ soln. During the experiment CO_2 equivalent to 12·77 ml. of 0·25N-$Ba(OH)_2$ was obtained, only about 10% of which was released at the lower temp. Assuming a mol.wt. of 308 for the hydrochloride, this represents 98% of one mol.prop. of CO_2. No crystalline product could be recovered from the quinoline.

Oxidation of sclerazinic acid with potassium permanganate: isolation of berberonic acid. Sclerazinic acid (0·51 g.) was dissolved by shaking in water (50 ml.) with a little $NaHCO_3$. Powdered $KMnO_4$ was slowly added with shaking until the colour was no longer discharged in 1 hr. The hydrated MnO_2 was removed by filtration and to the filtrate N-H_2SO_4 was added until it was just acid to Congo red. The colour darkened to orange. The solution was evaporated *in vacuo* nearly to dryness four times, water being added before the distillations subsequent to the first. The volatile acid amounted to 11·24 ml. of 0·1N (60% of 1 mol. prop.). This was converted into the *p*-bromophenacyl ester in the usual manner. Crystals of m.p. 85–86° were obtained, which did not depress the m.p. of authentic *p*-bromophenacyl acetate. Therefore the volatile acid produced in the reaction is acetic acid.

The non-volatile aqueous residue was extracted with ether in a continuous extractor for 24 hr. The crystalline solid obtained (0·28 g.) on removal of the ether had m.p. 233° (decomp., frothing). Recrystallization from water (decolorizing charcoal) gave compact prisms, m.p. 238–240° (decomp.). The product contained no chlorine, gave with $FeCl_3$ a white or very light-yellow ppt. and with $FeSO_4$ a port-wine colour (Found: C, 42·1; H, 3·5; N, 6·05. $C_8H_5O_6N,H_2O$ requires C, 41·9; H, 3·1; N, 6·1%). A sample of berberonic acid prepared from berberine nitrate by oxidation with nitric acid, when recrystallized from water had m.p. 241–242° (decomp.), and there was no depression on admixture with the product of oxidation with $KMnO_4$. Hence the latter is berberonic acid.

For confirmation the *p*-bromophenacyl esters of the two acids were prepared. The ester from the product of oxid-

ation with $KMnO_4$ had m.p. 175–176° after recrystallization from ethanol (microrosettes of needles) (Found: C, 48·3; H, 2·7; N, 1·85; Br, 29·1. $C_{32}H_{20}O_8NBr_3$ requires C, 47·9; H, 2·5; N, 1·75; Br, 29·9%). The *p*-bromophenacyl ester of authentic berberonic acid had the same m.p., 175–176°, and there was no depression on mixing.

Action of methylamine on sclerotiorin: N-methylsclerotiorazin. Sclerotiorin (0·25 g.) was treated with aqueous methylamine (33%, w/v; 5 ml.). The substance immediately became dark red but complete solution was not attained. The mixture was well stirred and all lumps were broken up. After 15 min., water (25 ml.) was added, the mixture was made just acid to Congo red and the solid (0·22 g.) was collected. Crystallization from aq. methanol (50%, v/v) furnished an *N-methylsclerotiorazin* as purple-black needles, m.p. 220–222° (Found: C, 65·3; H, 6·3; N, 3·8; Cl, 8·8. $C_{22}H_{26}O_4NCl$ requires C, 65·4; H, 6·5; N, 3·5; Cl, 8·8%).

Reduction of N-methylsclerotiorazin. N-Methylsclerotiorazin (0·93 g.) in acetic acid (40 ml.) was well stirred during the addition of Zn dust (1 g.). The colour of the solution changed from deep red to orange. The reaction was considered complete when no further lightening occurred (about 20 min.). The solution was filtered to remove residual Zn and poured into water. The gelatinous ppt. produced gradually crystallized in sheaves of needles. The product (0·74 g., m.p. 239°) formed golden-yellow needles of *deacetyldihydro-N-methylsclerotiorazin*, m.p. 239–241°, on recrystallization from methanol (Found: C, 65·9, 66·0; H, 7·4, 7·3; N, 3·5; Cl, 9·85, 9·9. $C_{20}H_{26}O_3NCl$ requires C, 66·0; H, 7·2; N, 3·85; Cl, 9·7%).

N-Methylsclerotiorazin (2 g.) was dissolved in methanol and reduced in presence of Zn dust (2 g.) and H_2SO_4 (50%, v/v; 2 ml.). The product was poured into water and the filtrate was distilled in the usual manner for collection of volatile acids. Titration of the distillate revealed that 46·8 ml. of 0·1 N-volatile acid had been produced (94% of 1 mol.prop.). Conversion into the *p*-bromophenacyl ester afforded a product of m.p. 84–85°, not depressed on admixture with *p*-bromophenacyl acetate.

SUMMARY

1. The product of the reaction of sclerotiorin with ammonia, $C_{21}H_{24}O_4NCl$, named sclerotiorazin, is readily reduced by zinc and acetic acid, yielding sclerotazin ($C_{19}H_{22}O_2NCl$) and acetic acid.

2. By mild alkaline hydrolysis of sclerotazin the chlorine atom is eliminated and replaced by a hydroxyl group, affording hydroxydechlorosclerotazin, $C_{19}H_{23}O_3N$.

3. Sclerotazin forms a diacetate, from which the unsaturated side chain is removed by ozonolysis and replaced by a carboxyl group, affording the diacetate of sclerazinic acid $C_{11}H_8O_4NCl$.

4. Oxidation of sclerazinic acid with alkaline potassium permanganate solution gives berberonic acid. From these degradations a tentative partial molecular structure for sclerotazin is deduced.

5. Sclerotiorin reacting with methylamine produces *N*-methylsclerotiorazin ($C_{22}H_{26}O_4NCl$), which is reduced by zinc and acetic acid to deacetyldihydro-*N*-methylsclerotiorazin, $C_{20}H_{26}O_3NCl$.

We wish to thank Imperial Chemical Industries Ltd., Akers Research Laboratories, The Frythe, Welwyn, Herts, for carrying out some of the elementary analyses. The Uvispek Spectrophotometer used in this work was purchased by means of a grant from the Central Research Fund of London University.

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Comparative Detoxication

5. CONJUGATION OF AROMATIC ACIDS IN REPTILES: FORMATION OF ORNITHURIC ACID, HIPPURIC ACID AND GLUCURONIDES*

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The detoxication of aromatic acids in the animal body may involve condensation with a variety of compounds in different species and, as a rule, more than one type of conjugation is found in each species. Glycine conjugation occurs in most mammals (Williams, 1947), in frogs (Bunge & Schmiedeberg, 1876; Komori, Sendju, Sagara & Takamatsu, 1926), in some birds (Wu Chang & Johnson, 1957) and in insects (Smith, 1955). Ester glucuronides can also be formed in mammals (Williams, 1947) and in the chick (Wu Chang & Johnson, 1957; B. C. Baldwin, D. Robinson & R. T. Williams, unpublished work). Glutamine conjugations, which have been thought peculiar to

* Part 4: Smith (1955).

man and the chimpanzee, have now also been reported in rats, rabbits and chickens (Hiratani, 1957). The conjugation of xanthurenic acid with serine has been reported in rats (Rothstein & Greenberg, 1957). The ornithine conjugation has hitherto been found only in birds, where ornithuric acids result from the detoxication of benzoic acid (Jaffe, 1877).

It has sometimes been suggested that reptiles might also form ornithuric acids since other aspects of their nitrogen metabolism are similar to those of birds (Baldwin, 1948; Sahyun, 1948). The limited data available, however, appear to indicate the operation of the more usual glycine conjugation in reptiles. Thus Komori *et al.* (1926) isolated hippuric acid from the urine of an unidentified chelonian after feeding sodium benzoate. In an early report Schiff (1859) also stated that hippuric acid crystals had been recognized in the urine of *Testudo tabulata*. Khalil (1947, 1948*a*, *b*, 1951) has quoted analyses for hippuric acid, measured by extraction with ether and nitrogen analysis, in the normal urine of the snakes *Zambenis diadema* and *Eryx thebaicus*, the turtle *Chelone mydas* and the lizard *Chalcides ocellatus*.

A number of aromatic acids have now been fed to six species of reptile and it is shown below that, while in some cases a small amount of glycine conjugation occurs, the major amino acid conjugate excreted is an ornithuric acid.

MATERIALS AND METHODS

Reference compounds. L(+)-Ornithine was prepared from L(+)-arginine by enzymic hydrolysis (Hunter, 1939) and treated, in N-Na_2CO_3 solution, with benzoyl chloride to give L(+)-ornithuric acid (N^2N^5-dibenzoylornithine), m.p. 183°, $[\alpha]_D^{25}$ 9·7°. DL-Ornithuric acid (m.p. 185°) was similarly prepared from DL-ornithine.

L(+)-Ornithine was shaken with a solution of *p*-nitrobenzoyl chloride in toluene and a slight excess of N-Na_2CO_3 at 0° till the purple colour faded. Acidification with conc. HCl gave L(+)-N^2N^5-*bis*-(p-*nitrobenzoyl*)*ornithine* (*pp'*-dinitro-ornithuric acid), which after crystallization from ethanol had m.p. 100°, solidifying and remelting at 150–160°, $[\alpha]_D^{25}$ 6·0° in 0·1 N-NaOH (*c*, 1) (Found: C, 52·5; H, 4·8; N, 11·9. $C_{19}H_{18}O_8N_4$,Et·OH requires C, 52·9; H, 5·1; N, 11·8%). It had $\lambda_{max.}$ 270 mμ and $\epsilon_{max.}$ 24 000 in 0·1 N-NaOH or 0·1 N-HCl.

DL-N^2N^5-*Bis*-(p-*nitrobenzoyl*)*ornithine*, prepared similarly from DL-ornithine and crystallized from ethyl acetate and dilute acetic acid, had m.p. 193° (Found: C, 52·7; H, 4·2; N, 12·8. $C_{19}H_{18}O_8N_4$ requires C, 53·0; H, 4·2; N, 13·0%).

L(+)-N^2N^5-Bis-(*p*-nitrobenzoyl)ornithine (1·1 g.) in 100 ml. of H_2O was heated on a steam bath for 0·5 hr. with $FeSO_4$,$7H_2O$ (7·2 g.) and K_2CO_3 (4·1 g.). The crystals (0·8 g.) which formed on cooling the filtrate were recrystallized from water and the L(+)-N^2N^5-*bis*-(p-*aminobenzoyl*)-*ornithine* (*pp'*-diamino-ornithuric acid) had m.p. 182°, $[\alpha]_D^{25}$ 7° in 0·1 N-NaOH (*c*, 1) (Found: C, 60·4; H, 5·9; N, 15·1. $C_{19}H_{22}O_4N_4$,0·5H_2O requires C, 60·1; H, 6·1; N, 14·8%).

DL-N^2N^5-*Bis*-(p-*aminobenzoyl*)*ornithine*, m.p. 191°, was similarly prepared from the inactive nitro compound (Found: C, 60·4; H, 5·8; N, 14·8. $C_{19}H_{22}O_4N_4$,0·5H_2O requires C, 60·1; H, 6·1; N, 14·8%). It had $\lambda_{max.}$ 273, $\epsilon_{max.}$ 32 000 in 0·1 N-NaOH and $\lambda_{max.}$ 222, $\epsilon_{max.}$ 22 000 in 0·1 N-HCl.

DL-N^5-Benzyloxycarbonylornithine (Harris & Work, 1950) was dissolved in a slight excess of N-Na_2CO_3 at 0° and shaken with a solution in toluene of an equivalent amount of *p*-nitrobenzoyl chloride until the purple colour faded, the solution being kept at pH 10–11 with additions of 2 N-NaOH. The precipitate which formed on acidification with conc. HCl was crystallized from methanol, and then ethanol, to give DL-N^5-*benzyloxycarbonyl*-N^2-(p-*nitrobenzoyl*)*ornithine*, m.p. 150–152° (Found: N, 9·8%; equiv. 412. $C_{20}H_{21}O_7N_3$ requires N, 10·1%; equiv. 415). This compound (2 g.) was reduced with H_2 and Adams Pd catalyst (0·2 g.) in 1:1 (v/v) aqueous methanolic solution (200 ml.). Concentration gave a crystalline precipitate (0·9 g.), which was recrystallized from H_2O. The DL-N^2-(p-*aminobenzoyl*)*ornithine* had m.p. 190° (decomp.) (Found: C, 53·1; H, 6·9; N, 15·7. $C_{12}H_{17}O_3N_3$,H_2O requires C, 53·5; H, 7·1; N, 15·6%).

DL-N^5-(p-*Nitrobenzoyl*)*ornithine*, m.p. 220° (Found: C, 51·4; H, 5·2; N, 14·9. $C_{12}H_{15}O_5N_3$ requires C, 51·2; H, 5·4; N, 14·9%), and DL-N^5-(p-*aminobenzoyl*)*ornithine*, m.p. 215° (Found: C, 53·4; H, 7·0; N, 15·0. $C_{12}H_{17}O_3N_3$,H_2O requires C, 53·5; H, 7·1; N, 15·6%), were prepared as described by McGilvery & Cohen (1950) for the preparation of the corresponding optically-active compounds.

DL-Ornithine dissolved in N-Na_2CO_3 was shaken with salicylic azide, the solution being maintained at pH 10–11 by adding 2 N-NaOH. After 0·5 hr. the solution was acidified with conc. HCl, and the precipitate removed, washed with $CHCl_3$ and crystallized from a large volume of water, in which it was sparingly soluble. The DL-N^2N^5-*disalicyloylornithine* (*oo'*-dihydroxyornithuric acid) had m.p. 173° after drying over P_2O_5 (Found: C, 61·2; H, 5·2; N, 7·9. $C_{19}H_{20}O_6N_2$ requires C, 61·3; H, 5·4; N, 7·5%).

p-Aminobenzoylglucuronide was prepared biosynthetically (cf. Bray, Lake, Neale & Thorpe, 1948). *p*-Aminobenzoic acid (6 g.) was fed to three rabbits and the glucuronide gum obtained by the lead acetate procedure (cf. Kamil, Smith & Williams, 1951). This gum contained much free *p*-aminobenzoic acid, which crystallized on standing. The lead acetate separation was therefore repeated on the glucuronide gum and a second clear glucuronide gum (300 mg.) obtained free from *p*-aminobenzoic acid. This was treated with 100 mg. of *p*-anisidine in 2 ml. of water, when a crystalline precipitate of the p-*anisidine salt of* p-*aminobenzoyl glucosiduronic acid* was obtained (150 mg.). After crystallization from water it had m.p. 184°, $[\alpha]_D^{25}$ −5° in water (*c*, 1) (Found: C, 50·7; H, 5·8; N, 5·7. $C_{20}H_{24}O_9N_2$, $2H_2O$ requires C, 50·8; H, 6·0; N, 5·9%).

Another preparation of the glucuronide gum, after free *p*-aminobenzoic acid has been filtered off, was methylated and acetylated (cf. Kamil *et al.* 1951) and the product crystallized repeatedly from ethanol to give a small yield of *methyl* (p-*acetamidobenzoyl triacetylglucosid*)*uronate*, m.p. 205°, $[\alpha]_D^{25}$ −28·5° in $CHCl_3$ (*c*, 1) (Found: N, 3·2. $C_{22}H_{25}O_{12}N$ requires N, 2·8%).

Salicyluric acid, m.p. 168°, and *p*-nitrohippuric acid, m.p. 135°, were prepared by standard methods. *p*-Aminohippuric acid, m.p. 199°, was purchased.

Animals. Tortoises (*Testudo graeca*) which weighed about 700–900 g. were fed on cabbage and boiled potatoes and were kept over a funnel on wire gauze to collect the milky urine. A 100 w electric lamp suspended at one corner of the enclosure kept the temp. at 30–35°. Water tortoises (*Emys orbicularis*) which weighed about 350 g. were fed on raw fish and kept in an aquarium tank at 25° with a brick island at one end. For metabolic experiments they were transferred to a 12 in. diameter glass-covered dish containing 500 ml. of water which was removed daily, evaporated and examined for metabolites.

Grass snakes (*Natrix natrix*) which weighed about 50–100 g. were fed occasionally on fresh, chopped raw codfish and water and kept in a glass tank at 25° on a layer of gravel with some rough bricks. In metabolic experiments they were placed in covered 5 l. beakers from which any excreta were washed out daily.

Slow worms (*Anguis fragilis*) weighed about 25–50 g. and were treated in the same way as the snakes except that they were fed on small white garden slugs.

Green lizards (*Lacerta viridis*) weighed about 50 g. and were kept in a dry, open, glass tank containing a hollow brick and kept at 25–30° by a suspended electric-light bulb. They were fed on mealworms and water. In metabolic experiments they were kept individually in 5 l. beakers from which any excreta were washed out daily.

Young alligators (*Caiman* sp.) weighed 100–150 g. and were kept in a glass-covered glass tank in about 3 cm. of water which was kept at 30° by an electric lamp suspended over the surface. A hollow brick was provided near the lamp as an island and cave. They were fed on earthworms. In qualitative metabolic experiments urine was withdrawn from the cloaca by blood pipette. In quantitative experiments, a glass funnel having a 15 cm. diameter wire-gauze grid was set up inside the aquarium tank so that the crocodiles could be kept in a water-saturated atmosphere. Water (2–3 ml.) was given by mouth daily, and with this arrangement urine could be collected for periods up to 7 days.

Administration of compounds. These were given by subcutaneous injection as their sodium salts or as the free acids orally in gelatin capsules.

Paper ionophoresis. Compounds were applied as 2 mm. diameter spots to 8 cm. × 50 cm. strips of Whatman no. 1 paper moistened with electrolyte. These strips were clamped between 45 cm. sheets of plate glass and the ends dipped into reservoirs of electrolyte containing carbon electrodes. A potential difference of 500 v was applied across 45 cm. of paper. When 0·02 N-NaOH, 0·02 N-HCl or Britton & Robinson (1931) buffer (British Drug Houses Ltd.) was used, the current was about 1 mA/cm. width of paper. For identifications the 'unknowns' were run side by side with reference compounds for periods of 1–2 hr. No significance could be attached to the absolute directions of migration since no allowance was made for electro-osmotic effects. Migrations are therefore quoted in Table 2 relative

Table 1. *R_F values of some acids and their conjugated derivatives*

Whatman no. 1 or no. 4 paper was used; the chromatogram was run till the front had moved about 12–15 in. *A*, Butanol–acetic acid–H_2O (4:1:5); *B*, propanol–NH_3 (sp.gr. 0·88) (7:3); *C*, butan-2-one saturated with water; *D*, butan-2-one–2 N-NH_3 (1:1); *E*, butan-2-one–2 N-acetic acid–water (200:1:100); *F*, benzene–acetic acid–water (1:1:2); *G*, benzene–butanol–NH_3 (sp.gr. 0·88) (2:5:2). All proportions are by volume.

	Solvent						
	A	*B*	*C*	*D*	*E*	*F*	*G*
Benzoic acid	0·90	0·74	0·95	0·13	—	0·95	0·30
Hippuric acid	0·85	0·69	0·35	0·08	—	0·15	0·20
Ornithuric acid	0·95	0·93	0·50	0·38	—	0·30	0·60
Salicylic acid	0·90	0·81	0·90	0·42	—	0·90	0·40
Salicyluric acid	0·80	0·44	0·25	0·01	—	0·50	0·06
Disalicyloylornithine	0·95	0·59	0·80	0·01	—	0·23	0·19
p-Nitrobenzoic acid	0·95	0·75	0·50	0·37	—	0·80	0·30
p-Nitrohippuric acid	0·85	0·68	0·40	0·16	—	0·15	0·20
Bis-*p*-nitrobenzoylornithine	0·97	0·86	0·20	0·35	—	0·02	0·45
p-Aminobenzoic acid	0·90	0·32	0·85	—	0·90	0·80	0·05
p-Aminohippuric acid	0·67	0·34	0·10	—	0·26	0·05	0·05
N^5-(*p*-aminobenzoyl)ornithine	0·30	0·39	0·02	—	0·02	0·05	0·15
N^2-(*p*-aminobenzoyl)ornithine	0·33	0·25	0·01	—	0·01	0·05	0·05
N^2N^5-Bis-(*p*-aminobenzoyl)ornithine	0·73	0·47	0·10	—	0·45	0·05	0·05
p-Aminobenzoylglucuronide	0·43	0·31	0·05	—	0·05	0·05	0·05

Table 2. *Ionophoretic separation of some* p*-aminobenzoic acid derivatives*

The figures represent migration in centimetres, relative to that of *p*-aminobenzoic acid, to anode (+) or cathode (−) in 1 hr. Whatman no. 1 paper, 12 v/cm., was used.

	0·02 N-HCl	pH 3*	pH 4*	pH 5*	0·02 N-NaOH
p-Aminobenzoic acid	0	0	0	0	0
p-Aminohippuric acid	0	+0·1	+2·2	+0·1	−1·0
p-Aminobenzoylglucuronide	+3·0	+1·6	+2·2	−0·5	−1·5
Bis-*p*-aminobenzoylornithine	−0·8	0	−0·6	−1·0	−2·5
N^2-*p*-Aminobenzoylornithine	−2·9	−0·9	+1·0	−3·5	−4·0
N^5-*p*-Aminobenzoylornithine	−2·0	−0·7	+0·4	−3·0	−2·5

* Britton & Robinson (1931) buffer mixture.

to that of *p*-aminobenzoic acid to show the degree of separations achieved.

Paper chromatography. This was carried out by downward displacement as in earlier papers (e.g. Smith, Smithies & Williams, 1953) and approximate R_F values are quoted in Table 1 to indicate degrees of separation achieved. Identifications were made by running reference compound and unknown on the same paper in at least four of the solvents of Table 1.

Large-scale partition chromatography. When this was carried out on sheets of Whatman no. 3 MM or no. 1 paper the solutions were applied as bands 2 cm. wide across the width of the paper. Partition columns were also used. In the latter, diatomaceous earth (Hiflo Supercel, Johns Manville Co. Ltd.) was rubbed with approximately its own weight of the lower layer of a partition system to give a dry, crumbly powder. This was suspended in the top layer of the solvent mixture and the slurry poured into a glass tube plugged loosely at the lower end with cotton wool. After being allowed to settle, the column was washed with one column-length of upper layer and the lower end stoppered when the solvent reached the level of the filling. Mixtures for separation were dissolved in a little lower solvent layer and stirred with enough Hiflo Supercel to give a dry powder. This mixture was placed on top of the column and development with upper solvent layer started. Volumes of eluate were collected periodically and tested on paper chromatograms to see the progress of the separation.

Detection of compounds on paper. Colour reactions and fluorescence phenomena described previously (Friedler & Smith, 1954) were used. In general the ornithine conjugates behaved in these tests in the same way as the glycine derivatives. Hippuric acids were also detected by the orange or red colours given by the specific reaction of Gaffney, Schreier, Di Ferrante & Altman (1954) and aromatic amino compounds were revealed as yellow spots when sprayed with a mixture of equal volumes of 1% (w/v) ethanolic *p*-dimethylaminobenzaldehyde and 0·5N-HCl. Both N^2- and N^5-*p*-aminobenzoylornithines were detected as purple spots by the ninhydrin reagent.

Quantitative measurements. Lizards were kept in covered 5 l. beakers at 30–35° and the excreta washed out periodically into 25 ml. graduated flasks with aq. 0·1N-NH_3.

Alligators were kept as described above, the urine being diluted to 25 ml. Small volumes (usually 0·1–0·2 ml.) were separated on Whatman no. 3 MM paper in solvent *E* and the zones corresponding to different metabolites eluted chromatographically with water and assayed by the Bratton & Marshall (1939) method (cf. Friedler & Smith, 1954). Calibration curves were constructed for each metabolite, using the Unicam SP 600 spectrophotometer, and recoveries of known amounts of material, after chromatography and elution, ranged from 90 to 100%.

RESULTS

Chromatographic experiments

The acids were administered to the reptiles at a dose level of 0·2 mg./g. and excreta collected as described above. Spots of liquid urine (tortoises, alligators) were chromatographed directly. Solid excreta (snakes, lizards) were stirred to a cream with N-NH_3 and the liquid was filtered before chromatography. Results of chromatographic identifications are summarized in Table 3. In some experiments, where trace amounts could not be identified with certainty, large-scale paper chromatograms were run, appropriate zones were eluted and the concentrated eluates examined on further chromatograms.

The elimination of excretory products was slow and irregular. Diazotizable amines were detectable in snake excreta 6 weeks after dosing with *p*-aminobenzoic acid and in lizards after 3 weeks. Metabolites other than hippuric acids or ornithuric acids were present in the excreta after each aromatic acid had been fed but these were further investigated only in experiments with *p*-aminobenzoic acid (see below).

Isolation of hippuric and ornithuric acid from tortoise urine

A tortoise (*Testudo graeca*) weighing 900 g. was given 50 mg. of benzoic acid by mouth and kept on a funnel for 24 hr. The urine (10 ml.) reduced Benedict solution and gave a positive naphtharesorcinol reaction. It was evaporated to dryness *in vacuo* and extracted with a little aq. 2N-NH_3.

Table 3. *Paper-chromatographic identification of conjugates of aromatic acids in reptile urines*

		Detected in urine of					
Acid fed	Metabolite detected	Snake	Green lizard	Slow worm	Alligator	Tortoise	Water tortoise
Benzoic acid	Hippuric acid	Trace	–	–	.	+	+
	Ornithuric acid	+	+	+	.	+	+
Salicylic acid	Salicyluric	.	.	.	.	+	.
	Disalicyloylornithine	.	.	.	.	+	.
p-Nitrobenzoic acid	*p*-Nitrohippuric acid	.	Trace	.	.	.	.
	Bis-*p*-nitrobenzoylornithine	.	+	.	.	.	.
	p-Aminobenzoic acid	.	+	.	.	.	+
	Bis-*p*-aminobenzoylornithine	.	+	.	.	.	+
p-Aminobenzoic acid	*p*-Aminohippuric acid	Trace	Trace	.	+	+	.
	Bis-*p*-aminobenzoylornithine	+	+	.	+	+	.
	N^5-*p*-Aminobenzoylornithine	+	+	.	+	+	.
	p-Aminobenzoylglucuronide	+	+	.	+	+	.

Table 4. *Excretion of* p-*aminobenzoic acid and conjugates in* Lacerta viridis *and* Caiman *sp.*

	Percentage of dose excreted as *p*-amino-								
	-ornithuric acid			-hippuric acid			-benzoic acid		
Lacerta viridis	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
Days 1 and 2	9·2	3·1	9·8	.	.	.	3·4	1·0	2·6
Days 3 and 4	11·9	14·3	3·1	.	.	.	3·9	2·2	1·7
Days 5 and 6*	1·4	9·8	2·2	.	.	.	1·1	1·3	0·6
Totals	22·5	27·2	15·1	.	.	.	8·4	4·5	4·9
Caiman sp.									
Days 1–6*	11·8	17·1	.	3·5	9·3	.	4·4	5·5	.

* Excretion still incomplete.

After insoluble material had been filtered off the solution was applied as a strip 2 cm. × 30 cm. on Whatman no. 1 paper and separated in solvent *G*. Bands which quenched the paper fluorescence, corresponding to the R_F values of hippuric acid and ornithuric acid, were cut out and eluted chromatographically with water. Acidification of the ornithuric acid eluate gave a precipitate which was crystallized from water (13 mg.). This had m.p. 184°, not depressed by mixture with authentic L-ornithuric acid, and $[\alpha]_D^{25}$ 9·3 in 0·1 N-NaOH (*c*, 1·5) (Found: C, 67·2; H, 5·9; N, 8·0. $C_{19}H_{20}O_4N_2$ requires C, 67·0; H, 5·9; N, 8·2%). In another experiment in which 200 mg. of sodium benzoate was injected subcutaneously, 18 mg. of L-ornithuric acid was isolated from the first day's urine.

The hippuric acid zone on a large-scale paper chromatogram in solvent *G* was eluted with water and rechromatographed in solvent *F*. The eluate of the zone giving the colour reactions of Gaffney *et al.* (1954) gave a very small precipitate on acidification, which after crystallization from water had m.p. 188° (0·2 mg.), not depressed by mixing with hippuric acid.

In a similar experiment with the water tortoise (*Emys*) 100 mg. of benzoic acid was given orally and the first day's excretion examined. L-Ornithuric acid (1 mg.), m.p. and mixed m.p. 183°, and benzoic acid (3 mg.), m.p. and mixed m.p. 122°, were isolated.

Conjugation of p-*aminobenzoic acid*

Quantitative experiments. The acid was given orally to lizards and alligators at a dose level of 0·5 mg./g. The results are summarized in Table 4. *p*-Aminohippuric acid was present only in traces in lizard excreta and was not estimated. No evidence of any significant amount of acetylation was found in any of the reptiles in any qualitative experiment, and acid hydrolysis of urines in the quantitative experiments gave no increase of diazotizable amine.

The excreta of the reptiles in these quantitative experiments was usually entirely urine but in some experiments with alligators this was contaminated with faeces. In these experiments the diazotizable amino compound present was almost entirely *p*-aminobenzoic acid. Qualitative experiments showed that alligator faeces, which appeared to consist mainly of the earth from the earthworm diet, enzymically hydrolysed bis-*p*-aminobenzoylornithine to *p*-aminobenzoic acid; a small amount of N^5-*p*-aminobenzoylornithine was also produced. Prolonged exposure of the urine to the faeces resulted in the disappearance of any diazotizable amine. Experiments with obvious faecal contamination were therefore rejected.

Identification of ornithine conjugates. Six green lizards were each given 25 mg. of *p*-aminobenzoic acid by mouth and the excreta were collected for 6 days. The excreta were roughly ground and extracted with 20 ml. of boiling water for 30 sec. The filtrate was evaporated to dryness *in vacuo* and the residue separated on a partition column 2 cm. diam. × 50 cm. with solvent system *E*. Eluates containing material behaving like *p*-aminobenzoic acid and bis-*p*-aminobenzoylornithine on test paper chromatograms were collected.

The eluates containing bis-*p*-aminobenzoylornithine were evaporated to small bulk and further purified by large-scale chromatography in solvent system *A*. Insufficient material was obtained for preparing solid derivatives but the product behaved identically with bis-*p*-aminobenzoylornithine in each of the solvent systems of Table 1 and at each of the pH values of Table 2 in ionophoresis experiments. Hydrolysis with N-HCl at 100° for 4 hr. gave a mixture containing *p*-aminobenzoic acid, ornithine and N^2- and N^5-*p*-aminobenzoylornithines, which were detected chromatographically.

Material remaining on the original partition column was eluted with 500 ml. of water. After evaporation, the residue gave an intense naphtharesorcinol reaction and reduced Benedict solution. It was separated by large-scale chromatography on paper in solvent *E* into two fractions *X* and *Y*. The slower-moving one (*Y*) was eluted and was identified as N^5-*p*-aminobenzoylornithine by paper chromatography and ionophoretic experiments. No N^2-*p*-aminobenzoylornithine was found.

In other experiments, bis-*p*-aminobenzoylornithine and small amounts of N^5-*p*-aminobenzoylornithine were separated and identified in the excreta of *Natrix*, *Testudo* and *Caiman* by the methods described above.

Identification of p-*aminobenzoylglucuronide*. The faster-moving band (*X*) on the large-scale paper chromatogram in solvent *E* was eluted and behaved identically with *p*-aminobenzoylglucuronide when chromatographed in the solvent systems of Table 1. It also migrated at the same speed as the glucuronide in the ionophoretic systems of Table 2.

The eluate, which gave an intense naphtharesorcinol reaction, was hydrolysed with locust-crop fluid (which contains a β-glucuronidase) or by boiling for 1 min. with N-NaOH, to give *p*-aminobenzoic acid and glucuronic acid, which were identified on paper chromatograms. Quantitative naphtharesorcinol estimations of glucuronic acid (see Mead, Smith & Williams, 1958) gave values equivalent to a total of 14 mg. of *p*-aminobenzoylglucuronide in this eluate. The same value was also obtained by the Bratton & Marshall (1939) diazo estimation by using a calibration curve prepared from authentic *p*-aminobenzoylglucuronide. The eluate had an absorption maximum at 270 mμ in 0·1N-NaOH, with an intensity corresponding to the presence of 15·4 mg. of *p*-aminobenzoylglucuronide, if this was assumed to be the only absorbing material present.

In other similar experiments *p*-aminobenzoylglucuronide was identified in the excreta of *Natrix*, *Testudo* and *Caiman*. Identification was made, as above, by chromatography, ionophoresis and hydrolysis to *p*-aminobenzoic acid and glucuronic acid.

p-*Aminohippuric acid*. Only traces of *p*-aminohippuric acid were detected in the excreta of the lizards and snakes used. *Testudo* and *Caiman* excreted a greater proportion of this conjugate and excreta from these species were separated in large-scale paper chromatograms in solvent *E*. The band corresponding to *p*-aminohippuric acid was eluted and shown to contain material behaving identically with the reference compound in all the solvent systems used for chromatography and at each of the pH values used for ionophoresis. Hydrolysis of the eluate with N-HCl for 2 hr. at 100° gave *p*-aminobenzoic acid and glycine, which were identified on paper chromatograms (cf. Friedler & Smith, 1954).

DISCUSSION

Except for the data on reptiles quoted above, evidence for the existence of an ornithine conjugation is confined to birds. Information about birds is almost entirely concerned with the hen, which is well known to form ornithuric acids from ingested aromatic acids. Experiments with enzymes suggest that ducks and pigeons may also be capable of this conjugation (Schachter, Manis & Taggart, 1955; Efimochkina, 1951). Small amounts of a glycine conjugate may occur in the hen (Suga, 1919) and N^2- and N^5-substituted ornithines have been detected by Wu Chang & Johnson (1957) after nicotinamide had been fed to chicks. Similarly, small amounts of N^5-substituted ornithine and a glycine conjugate have been found in the reptile experiments.

All orders of terrestial vertebrates which have been investigated, including reptiles and birds, possess to some degree the ability to conjugate aromatic acids with glycine and glucuronic acid. It is only in reptiles and birds where the additional reaction with ornithine is possible, and it is of interest to consider the evolutionary relationship of these two groups to the rest of the vertebrates (Fig. 1). Present-day birds and reptiles are thought to have evolved from a different group of primitive reptiles from the reptilian ancestors of the modern mammals (see Young, 1950). Since amphibia, which branch off from the evolutionary tree at an earlier stage, use only glycine and glucuronic acid conjugations it seems possible that

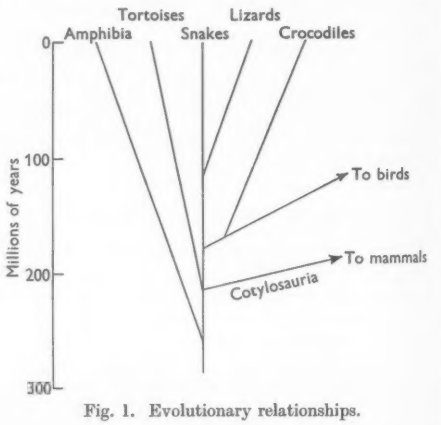

Fig. 1. Evolutionary relationships.

the ability to use ornithine in detoxication arose about 200 million years ago, when the cotylosaurian reptiles were becoming diversified into the ancestors of the later reptile orders.

There appears to be a relation between the presence of the ornithine conjugation and the excretion of waste nitrogen as uric acid. It may be significant that in the developing hen's egg the ornithine conjugation appears at the same time as the change to full uricotelic metabolism (Takahashi, 1928). Hens, the grass snake and the two species of lizard studied are all uricotelic animals (see Prosser, 1950, for summary) and it is in these species that the ornithine detoxication predominates over the formation of hippuric acids. The alligators and both aquatic (*Emys*) and terrestial (*Testudo*) tortoises are partly uricotelic and partly ureotelic. In these species the proportion of hippuric acids formed is much higher (e.g. Table 4) though the ornithuric acid is still the major metabolite.

Florkin (1949) has suggested that the mixed nitrogen metabolism in ancient orders like tortoises and crocodiles is a reflexion of their primitive nature, and he distinguishes them from the more recently evolved lizards and snakes. It is possible that the retention of a glycine-conjugating mechanism alongside the main ornithine detoxication is a similar relic of their amphibian ancestry.

The coincidence of uricotelism with an ornithine conjugation need not necessarily indicate any direct metabolic connexion between the two processes. The advantage of uric acid to organisms reproducing via a cleidoic egg has frequently been pointed out (e.g. Baldwin, 1948), but this in its turn may have introduced another biochemical problem. In the synthesis of uric acid by bird liver, four of the carbon atoms arise directly or indirectly from glycine, and the excretion of the daily intake of nitrogen must create a greater need for glycine in uricotelic than in ureotelic animals. It seems reasonable to believe that in the early stages of evolution of the reptilian ancestors of the birds the glycine-sparing action of an ornithine conjugation might have been an evolutionary asset.

Although ornithine conjugation has been found, so far, only in association with a uricotelic nitrogen metabolism, the converse is not true. Insects are primarily uricotelic but those species for which results are available form hippuric acids from aromatic acids (Smith, 1955*a*). The mechanism of the synthesis of uric acid in insects is not known; but it is of interest that, of the few insects whose requirements are known, two (*Aedes* and *Blatella*) require glycine as an essential amino acid (Levinson, 1955).

SUMMARY

1. Ornithuric acids have been detected as the major amino acid conjugate in the excreta of six species of reptile after feeding aromatic acids.

2. L(+)-Ornithuric acid has been isolated from excreta of *Testudo graeca* and *Emys orbicularis* dosed with benzoic acid.

3. After *p*-aminobenzoic acid was fed to *Natrix natrix*, *Lacerta viridis*, *Testudo graeca* and *Caiman* sp., small amounts of *p*-aminobenzoylglucuronide were detected in the excreta.

4. After feeding *p*-aminobenzoic acid, traces of *p*-aminohippuric acid were found in the excreta of *Natrix* and *Lacerta* and large amounts in the excreta of *Caiman* and *Testudo*.

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Countercurrent Distribution: Quantitative Analysis of Results, Especially where Separation is Incomplete

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A method has been described (Goodall, 1954) for calculating the amounts of each component in a mixture of two substances when separation in, say, a Craig machine, is incomplete. Pairs of tubes containing reasonable amounts of both substances are selected, and the amounts of the components found by solving sets of simultaneous equations. The possibility of extending the method to more than two components was also pointed out.

By an adaptation of standard statistical techniques it is now shown to be possible to calculate not only the composition of a mixture, but also the values of the partition ratios of the components if these are unknown, even when quite a small degree of separation has been achieved by countercurrent distribution. All the relevant tubes are used in one operation.

Apart from its obvious value in enabling information to be obtained in general countercurrent-distribution experiments, the method might occasionally have useful analytical applications. Thus for a pair of acids difficult to separate or to determine in the presence of each other, a system might be found for which a partial separation could be obtained in a relatively small number of countercurrent transfers. From a knowledge of the distribution ratio of each pure substance, and a measurement of the total acidity in each tube, the amount of each acid can be found. Examples are described below.

Bacher (1951) has described a simpler method from a different viewpoint for calculating partition ratios, by using all the relevant tubes in a zone, and has extended the method to the analysis of mixtures. The latter analysis, however, requires more than one measurement (e.g optical density at different wavelengths) on each tube. Bland, Hillis & Williams (1952) also discuss this method of finding partition ratios. These procedures, although less elaborate, are of less general application than those proposed here.

THEORETICAL

As is well known, when A units of a substance of partition ratio K_A have been submitted to n transfers in a countercurrent apparatus of the Craig type the amount of substance, Y_r, in tube r is given by the expression $Y_r = Aa_r$, where a_r is the appropriate term of the binomial distribution, which can be calculated (Williamson & Craig, 1947) or found from suitable tables (Eisenhart, 1952) when K_A and n are known. There will be a series of such equations, one for each tube. If Y_r is measured experimentally and A is unknown then a_r can be treated as an independent variable and Y_r as a dependent variable in the series of equations. The best estimate of A, when all the tubes containing measurable amounts of substance are used, is then found by the method of least squares. If A were known the true value of Y_r would be Aa_r, and the difference between this value and the experimental value of Y_r is $Y_r - Aa_r$. The principle of least squares states that the best estimate of A from the experimental data is the value which makes the sum of the squares of these differences, $\Sigma(Y_r - Aa_r)^2$, a minimum. By the usual process of differentiating and equating to zero we then get (removing the subscript r)

$$A = \Sigma Ya / \Sigma a^2. \qquad (1)$$

One can also calculate a sum of squares, R, representing the deviations of the experimental

values of Y from those calculated with the above estimated value of A. Thus

$$R = \Sigma Y^2 - A\Sigma Ya. \quad (2)$$

The standard deviation of the calculated values of Y is then given by

$$s = \sqrt{\frac{R}{N-1}}, \quad (3)$$

where N is the number of tubes used in the calculation (not n, the number of transfers). The standard deviation of A is given by

$$\text{S.D. of } A = \sqrt{\frac{R}{(N-1)\,\Sigma a^2}}. \quad (4)$$

In equations (1)–(4) the quantities Y and a are the actual quantities and not deviations from the means usually used in regression analysis. This implies that the regression line found passes through the point $a=0$, $Y=0$, as must in fact be the case.

If it is more convenient to analyse only one phase of the tubes then the same procedure is applied to these values. If the upper phase only is used, to get the total amount of A the result must be multiplied by $(1+K_A)/K_A$, where K_A is the partition ratio. For the lower phase only the appropriate factor is $1+K_A$.

If the correct value of the partition ratio for the substance has been used s (equation 3) and therefore R (equation 2) should not differ greatly from the value to be expected from the analytical method used to determine Y, plus any error due to mechanical imperfections in the distribution procedure. In most cases this latter error seems to be remarkably low, as shown by the excellent agreement between observed and calculated distributions on many occasions, even after several hundreds of transfers. To get the best estimate of the partition ratio of the substance therefore it is necessary only to find by trial the value which will make R a minimum. This will also give the most reliable estimate of A, which will be found to vary appreciably with change in the value of the partition ratio used for its calculation.

A convenient method for finding the best estimate of the partition ratio for a single substance with a relatively small number of transfers is to determine R for the nearest value of p $(=K/1+K)$ corresponding to the tube number in which the maximum of the zone occurs. R is then recalculated with values of p 0·01 units above and below the central value. This figure is chosen for convenience in using the tables of the binomial distribution (Eisenhart, 1952), where the entries are given at intervals of p differing by 0·01. These three values of R, which we will call R_0, R_{+1} and R_{-1} respectively, can then be assumed with sufficient accuracy to be related by a quadratic equation in which p is the independent variable. Since the actual values of p are equally spaced they can be replaced by the values $p'=+1$, 0 and -1 to simplify the calculations. We thus get an equation of the form

$$\alpha(p')^2 + \beta p' + \gamma = R, \quad (5)$$

where α, β and γ are constants. When $p'=0$, $\gamma = R_0$. When $p'=+1$ and -1 we get

$$\alpha + \beta + R_0 = R_{+1}$$

and

$$\alpha - \beta + R_0 = R_{-1}$$

respectively. From which

$$\alpha = \frac{R_{+1}+R_{-1}}{2} - R_0,$$

$$\beta = \frac{R_{+1}-R_{-1}}{2}.$$

We now differentiate the original equation (5) and equate to zero to find the value of p' which will make R a minimum, giving $2\alpha p' + \beta = 0$ or $p' = -\beta/2\alpha$.

Substituting the values of α and β in terms of the observed values of R we get the formula

$$p' = -\frac{1}{2}\left[\frac{R_{+1}-R_{-1}}{R_{+1}+R_{-1}-2R_0}\right], \quad (6)$$

where p' is 100 times the *change* in the value of p corresponding to $p'=0$ required to make R a minimum. For larger numbers of transfers not given in the tables tube number can be substituted for p, since $p=M/n$, where M is the tube number in which the maximum of the zone is found after n tranfers. It may be more convenient to calculate R_{+1} and R_{-1} for tubes at intervals of more than one tube away from the central value. So long as the intervals are equal the above formula (6) will apply, but a different factor will be required to convert back from p' into actual tube number.

A 'maximum-likelihood' estimate for the standard error of p' can be shown to be given by

$$\text{S.E. of } p' = \sqrt{\frac{R_m}{\alpha(N-1)}}, \quad (7)$$

where R_m is the minimum value of R. Figures obtained with equation (7) may be unreliable if the experimental errors in the original observations are not normally distributed. In view of the wide range of values from the tails to the peak of a zone frequently encountered, such departures from normality may well be considerable. Nevertheless, this standard error may be of some value as an objective criterion where the identification of a substance by its partition ratio is in question.

The assumption of a simple quadratic relation (5) is an approximation. Hence if the value of p'

giving a minimum were found to be outside the range +1 to −1 chosen, it would be in the nature of an extrapolation and it would be advisable for accuracy to take a further point in the direction indicated by the results. This is unlikely to occur with a single, clearly defined zone, but could happen in some of the more complex cases discussed below. If the minimum value of R thus found were judged to be much greater than would be justified by the likely experimental error, then an explanation must be sought, the most likely being the presence of more than one substance. Comparison of the graphs of observed and calculated values will then usually give some indication of the presence of such a substance, or substances, and at least a rough idea of their partition ratios. The results can then be further analysed as now described.

This treatment for a single zone is quite general and can be extended to any number of substances. Thus for a mixture containing A, B, C, D, etc., units of a series of substances in tube r we will have a total content Y_r, given by

$$Y_r = Aa_r + Bb_r + Cc_r + Dd_r + \ldots;$$

where a_r, b_r, c_r, d_r, ... are the appropriate binomial coefficients for each substance. Provided that a sufficient number of experimental values of Y_r are observed, estimates based on the least-squares principle can be obtained for each of the quantities A, B, C, D, etc. The sum of squares of the differences between the observed values, Y_r, and the true values will be given by the expression

$$\Sigma[Y_r - (Aa_r + Bb_r + Cc_r + Dd_r + \ldots)]^2.$$

This is differentiated and equated to zero with respect to each of the quantities A, B, C, D, etc., in turn. This leads to a symmetrical series of simultaneous equations:

$$\left.\begin{aligned} A\Sigma a^2 + B\Sigma ab + C\Sigma ac + D\Sigma ad + \ldots &= \Sigma Ya, \\ A\Sigma ab + B\Sigma b^2 + C\Sigma bc + D\Sigma bd + \ldots &= \Sigma Yb, \\ A\Sigma ac + B\Sigma bc + C\Sigma c^2 + D\Sigma cd + \ldots &= \Sigma Yc, \\ A\Sigma ad + B\Sigma bd + C\Sigma cd + D\Sigma d^2 + \ldots &= \Sigma Yd, \\ \text{etc.} & \end{aligned}\right\} \quad (8)$$

These equations can be solved for A, B, C, D, and so on. Matrix methods such as those given by Davies (1957) are recommended for this, since means for the easy calculation of standard errors are also provided. Hence, in theory at least, it should be possible to calculate the composition of any zone provided that the number of substances present, and their partition ratios, are known. Only two assumptions are made: (*a*) it is assumed that the partition ratios are independent of concentration and of the presence of other substances; and (*b*) sufficient separation is achieved to give changes in concentration in a reasonable number of tubes greater than the experimental error. In practice the actual separation need only be quite small for this to occur.

For a two-component mixture the above procedure leads to the formulae:

$$A = 1/\theta\ (\Sigma Ya.\Sigma b^2 - \Sigma Yb.\Sigma ab) \quad (9)$$

and

$$B = 1/\theta\ (\Sigma Yb.\Sigma a^2 - \Sigma Ya.\Sigma ab), \quad (10)$$

where

$$\theta = \Sigma a^2.\Sigma b^2 - (\Sigma ab)^2.$$

Residual sum of squares,

$$R = \Sigma Y^2 - A\Sigma Ya - B\Sigma Yb. \quad (11)$$

S.D. of calculated values of Y_r,

$$s = \sqrt{\frac{R}{N-2}}. \quad (12)$$

S.E. of A

$$= s\sqrt{\frac{\Sigma a^2}{\theta}}. \quad (13)$$

S.E. of B

$$= s\sqrt{\frac{\Sigma b^2}{\theta}}. \quad (14)$$

As already mentioned, actual values of Y, a, b, c, d, etc., and not deviations from the means, are used in the calculations. Further, since the means are not calculated, the number of degrees of freedom is one more than is given in the usual formulae used in multiple regression, e.g. $N-2$ and not $N-3$ in equation (12) above for a two-component mixture.

As for a single zone, if incorrect values of the partition ratio are used the value of R will be increased. Hence by trial with a mixture of components of unknown partition ratios the best values of these can be found. Since, in, for example, a two-component mixture, four constants are being estimated from the data a sufficient number of tubes (say about 10 for two components) must be measured to give a reasonable estimate of experimental error. A systematic method for finding these best values, with standard errors, can be adapted from one described in another connexion (Davies, 1956). In practical examples, however, it was usually found possible to guess probable values of the partition ratios close to the true ones by inspection of the usual graph of total tube content against tube number. Further, it was found that relatively small changes in the values of K usually produced marked changes in R, so that after calculations with a few trial values close to those guessed it was easy to select by inspection values of K which made R a minimum (see examples below). Unless an order of accuracy were being attempted which is not usually justified by experiments of this type further elaboration should not be necessary. It was found that the further the partition ratio, K, is from unity, the greater is the change in R for a given change in K. Incidentally it may be pointed out that $p\ (=K/1+K)$ is a more

generally useful constant to quote for a substance than the partition ratio. It is given directly by the ratio of the amount in the upper phase to the total in a single partition, and is the quantity required for entering the binomial tables, or for direct calculation from the bionomial-distribution formulae. For $K>1$ we use $q=1-p$, and reverse the order of numbering of the tubes. q is the ratio of the amount in the lower phase to the total in a single distribution. In calculations based on the approximation used for large numbers of transfers the quantity $nK/(1+K)^2$ frequently occurs and can be conveniently replaced by npq. p is also given directly by the ratio M/n, where M is the tube number of a zone maximum and n is the total number of transfers.

RESULTS

Example 1. A countercurrent distribution was carried out in these laboratories of an extract of blood from patients dosed with a certain drug. A single zone was obtained, and it was important to decide whether this zone represented a single substance, or a mixture of closely related substances. Such a decision could be made by known simpler methods (Weisiger, 1956; Bacher, 1951), but the results have been calculated in detail by the present method only as an illustration of procedure. The figures are shown in Table 1. The agreement between observed and calculated values of Y was actually quite close enough for the point at issue without further calculation. The only advantage of the more elaborate method is that it enables confidence limits to be given to the estimates.

Example 2. As a preliminary experiment with mixtures, quantities of glutaric and adipic acids were weighed out and were distributed between 25 ml. layers of mutually saturated butan-1-ol and water in stoppered bottles. The lower layers were transferred by means of a hypodermic syringe. The

Table 1. *Analysis of a single zone obtained from the countercurrent distribution of a biological extract*

Measurements are in units of optical density, 20 transfers.

Tube	Binomial coefficients (a) when			Concn. in each tube (Y)	
no.	$q=0{\cdot}37$	$q=0{\cdot}38$	$q=0{\cdot}39$	Obs.	Calc. (when $q=0{\cdot}38$)
8	0·021	0·025	0·030	0·068	0·105
9	0·047	0·054	0·062	0·205	0·233
10	0·087	0·097	0·107	0·393	0·418
11	0·135	0·144	0·152	0·624	0·621
12	0·173	0·177	0·179	0·761	0·763
13	0·181	0·177	0·172	0·774	0·763
14	0·154	0·145	0·134	0·627	0·625
15	0·105	0·094	0·084	0·430	0·405
16	0·056	0·048	0·041	0·225	0·207
17	0·022	0·018	0·015	0·060	0·078

Details of calculations for $q=0{\cdot}38$:

$$\Sigma a^2=0{\cdot}128833; \quad \Sigma Ya=0{\cdot}555657; \quad \Sigma Y^2=2{\cdot}400925.$$

$$A=\frac{\Sigma Ya}{\Sigma a^2}=4{\cdot}313\pm0{\cdot}061$$

$$R=\Sigma Y^2-A\Sigma Ya=0{\cdot}004376$$

$$\text{S.E.}=\sqrt{\frac{0{\cdot}004376}{9}}=\pm0{\cdot}022$$

$$\text{S.E. of } A=\sqrt{\frac{0{\cdot}004376}{9\times0{\cdot}128833}}=\pm0{\cdot}061$$

For $q=0{\cdot}37$, $R=0{\cdot}006215$

For $q=0{\cdot}38$, $R=0{\cdot}004376$

For $q=0{\cdot}39$, $R=0{\cdot}023841$

$$p'=-\frac{1}{2}\left(\frac{0{\cdot}006215-0{\cdot}023841}{0{\cdot}006215+0{\cdot}023841-2\times0{\cdot}004376}\right)$$

$$=0{\cdot}41.$$

Therefore the best estimate of $q=0{\cdot}3759$; $K_A=1{\cdot}660$.

partition ratios (butan-1-ol/water) found in tap funnels were glutaric acid $K = 1{\cdot}99$; $p = 0{\cdot}33$; adipic acid $K = 3{\cdot}82$; $p = 0{\cdot}21$. In order to distribute the material uniformly over the six bottles nine transfers were applied, the extra aqueous layers from the end bottle being discarded. After addition of sufficient ethanol to give a homogeneous solution the contents of each bottle were titrated with 0·1 N-NaOH solution with bromothymol blue as indicator. The results are shown in Tables 2 and 3.

It is seen that the values found agree with the true values to within about 1–2 %. The standard errors quoted are calculated on the assumption that the partition ratios used are correct and constant. The number of observations, and the accuracy being attempted here, were scarcely sufficient to justify further trials to improve the results with improved partition ratios.

Example 3. Studies of the separation of porphyrins by countercurrent distribution are described by Granick & Bogorad (1953). Fig. 5 of their paper illustrates the distribution (156 transfers) of haematoporphyrin dihydrochloride (25 mg.), deuteroporphyrin dihydrochloride (25 mg.) and mesoporphyrin (22 mg.). The results suggest the presence of an impurity between the deuteroporphyrin and mesoporphyrin zones. The presence of such a substance was confirmed by the authors by paper chromatography of some of the relevant fractions. Values from the graph were read off from tube 60 to tube 85 inclusive. Partition ratios deduced from the positions of the peaks were assumed for deuteroporphyrin ($K = 0{\cdot}576$) and mesoporphyrin ($K = 1{\cdot}33$). Analyses of the three-component system as described below, assuming the peak of the unknown substance to be at tube 70, 74 or 78, gave values for R, the residual sum of squares, of 0·2251, 0·1440 and 0·7117 respectively. From equations 6 and 7 the minimum value of R is found to correspond to a tube number of $72{\cdot}5 \pm 0{\cdot}5$. To confirm the effectiveness of the analysis, results were recalculated for tubes 72, 73 and 74, giving a minimum at tube $72{\cdot}6 \pm 0{\cdot}5$. (The small difference is probably due to the assumption of a simple quadratic relation.) Tube 73 (partition ratio 0·880), the nearest whole number, was taken as sufficiently close for the purpose, giving deuteroporphyrin $22{\cdot}2 \pm 0{\cdot}2$ mg. (22·0 mg.); mesoporphyrin $16{\cdot}5 \pm 0{\cdot}2$ mg. (16·4 mg.); unknown $3{\cdot}2 \pm 0{\cdot}1$ mg.; $R = 0{\cdot}1068$.

The values in parentheses are those calculated from measurements of the amounts in the tubes at the maxima of the zones. It is not certain from the original whether the low value for the deuteroporphyrin is due to impurities in the original sample, or to the fact that results on the diagram are given as the free base instead of as hydrochloride, but it is clear, as suggested in the paper, that impurities were present in the mesoporphyrin. An approximate result of similar magnitude can be obtained by subtracting from the totals in each tube the values for deuteroporphyrin and mesoporphyrin calculated from their maxima. The position of the maximum of the zone of the unknown is, however, uncertain over at least three tubes. If all three zones had overlapped more, such a method would be much less certain, or impossible, but this limitation does not apply to the regression method.

To illustrate the method the steps in the calculation, assuming a maximum for the unknown at tube 73, are as follows.

Total sum of square of concentrations, as read from the graph in cm., $\Sigma Y^2 = 112.3508$. Sum of squares of calculated coefficients for deuteroporphyrin (A, $K = 0{\cdot}576$) $\Sigma a^2 = 0{\cdot}012968$. Sum of squares of calculated coefficients for mesoporphyrin (B, $K = 1{\cdot}33$), $\Sigma b^2 = 0{\cdot}009615$. Sum of squares of calculated coefficients of unknown (C, $K = 0{\cdot}880$) $\Sigma c^2 = 0{\cdot}045068$.

The various sums of products are:

$$\Sigma Ya = 0{\cdot}981979,\ \Sigma ab = 0{\cdot}000028;$$
$$\Sigma Yb = 0{\cdot}575949,\ \Sigma ac = 0{\cdot}007293;$$
$$\Sigma Yc = 1{\cdot}347302,\ \Sigma bc = 0{\cdot}007324.$$

From these we get the series of equations (cf. equation 8):

$$0{\cdot}012968A + 0{\cdot}000028B + 0{\cdot}007293C = 0{\cdot}981979,$$
$$0{\cdot}000028A + 0{\cdot}009615B + 0{\cdot}007324C = 0{\cdot}575949,$$
$$0{\cdot}007293A + 0{\cdot}007324B + 0{\cdot}045068C = 1{\cdot}347302.$$

Table 2. *Distribution of a known mixture of glutaric and adipic acids between butan-1-ol and water*

Nine transfers.

Bottle no.	Total content in ml. of 0·1 N-NaOH	Binomial coefficients for glutaric acid when $p = 0{\cdot}33$	Binomial coefficients for adipic acid when $p = 0{\cdot}21$
0	11·5	0·027	0·119
1	30·5	0·121	0·287
2	39·2	0·238	0·305
3	31·4	0·274	0·189
4	18·5	0·202	0·075
5	7·7	0·099	0·020

Table 3. *Values calculated for glutaric and adipic acids from results shown in Table 2, compared with theory*

	Vol. of 0·1 N-NaOH (ml.)	
	Found (equations 9–14)	Theory
Glutaric acid	$59{\cdot}9 \pm 1{\cdot}0$	60·6
Adipic acid	$81{\cdot}2 \pm 1{\cdot}1$	80·3
Totals	141·1	140·9

The inverse of the matrix of coefficients of A, B and C is found to be (Davies, 1957):

88·99461	11·81154	−15·83524
11·81154	120·31949	−21·46440
−15·83524	−21·46440	28·23934

Then

$$A = 88{\cdot}99461 \times 0{\cdot}981979 + 11{\cdot}81154 \times 0{\cdot}575949 - 15{\cdot}83524 \times 1{\cdot}347302 = 69{\cdot}91302.$$

Similarly, $B = 51{\cdot}97772$; $C = 10{\cdot}13455$.

$$R = 112{\cdot}3508 - 69{\cdot}91302 \times 0{\cdot}981979 - 51{\cdot}97772 \times 0{\cdot}575949 - 10{\cdot}13455 \times 1{\cdot}347302 = 0{\cdot}1068$$ (cf. equation 11 for two components)

$$s = \sqrt{\frac{0{\cdot}1068}{23}}$$ (cf. equation 12. Here $N = 26$ and three constants have been determined)

$$= \pm 0{\cdot}068 \ (\equiv 0{\cdot}022 \text{ mg.; see below}).$$

The standard errors of A, B and C are given by $s\sqrt{88{\cdot}99461}$, $s\sqrt{120{\cdot}31949}$ and $s\sqrt{28{\cdot}23934}$ respectively, i.e. ±0·64, ±0·74 and ±0·36. The values under the square-root sign are the terms of the leading diagonal of the inverse matrix above.

The results are converted into the figures in milligrams given above by the relation: 4·40 cm. on the graph ≡ 1·40 mg. It should perhaps be pointed out that the various standard errors will include the uncertainties due to reading from the printed diagrams.

For calculation, assuming a different tube number for the maximum for the unknown, only those sums of squares and products involving C need to be recalculated.

For the most probable position of the maximum we call values of R for tubes 70, 74 and 78 R_{-1}, R_0, and R_{+1} respectively. We then have (equation 6):

$$p' = -\frac{1}{2}\left[\frac{0{\cdot}7117 - 0{\cdot}2251}{0{\cdot}7117 + 0{\cdot}2251 - 2 \times 0{\cdot}1440}\right] = -0{\cdot}375.$$

One unit of p' is equivalent to four tubes, so $p = 4 \times -0{\cdot}375 = -1{\cdot}500$, i.e. the maximum of the zone is about half-way between tubes 72 and 73. The minimum value of R calculated by substituting $p' = -0{\cdot}375$ in the fitted equation (5) = 0·0984, from which (equation 7) the standard deviation of $p' = \sqrt{\frac{0{\cdot}0984}{23 \times 0{\cdot}3244}} = \pm 0{\cdot}115$. The standard deviation of the tube number is four times this, i.e. ±0·460.

Example 4. Fried, Numerof & Coy (1952) used a 24-transfer distribution at one stage in the isolation and purification of some veratrum alkaloids. An analysis of the middle composite zone from their diagram was tried. The first 10 or 12 tubes contained a substance with a definite peak at tube 5 which overlapped the zone being analysed. Assuming a maximum at tube 5 the amounts of this substance present in each tube were calculated and subtracted from the totals found. (Alternatively, the analysis could have been applied to a four-component system on the uncorrected values, but would have been unnecessarily elaborate.) The correction was found to extend to tube 12 within the order of accuracy attempted. At the other end there was similar overlapping, possibly by more than one substance, so tubes up to no. 19 only were used. The main zone clearly consisted of at least three substances, and trial analyses were made as shown in Table 4. It is seen that small changes in any of the partition ratios have a very marked effect on R, and further refinement would require interpolation in the binomial tables.

In view of the uncertainties at the ends of the zone this refinement does not seem worth attempting, although the standard deviation for the best set (±0·02 g.) still seems rather large. There is also the possibility of still further substances being present in the zone. The object in presenting the figures is to point out that with the information obtained in this analysis further fractionation of this main zone

Table 4. *Analysis of results of Fried, Numerof & Coy* (1952)

Three substances (A, B and C) are assumed to be present.

	Substance	Assumed value of p	Weight calculated (equation 8) (g.)	Residual sum of squares (R) (cf. equation 11)
First trial	A	0·46	0·415	0·3768
	B	0·62	3·00	—
	C	0·71	0·693	—
Second trial	A	0·50	0·539	0·3875
	B	0·62	2·76	—
	C	0·71	0·801	—
Third trial	A	0·48	0·467	0·3679
	B	0·62	2·89	—
	C	0·71	0·746	—
Fourth trial	A	0·48	0·430±0·066	0·3030
	B	0·62	3·06 ±0·09	—
	C	0·73	0·632±0·076	—

by distribution methods could be planned as an alternative to fractional crystallization. Thus in a larger apparatus it can be calculated that about 200 transfers would be required for substantially complete separation of A and B, but the separation of B and C would require about 350 transfers. Alternatively, repeated distributions with regrouping of the side fractions with a smaller number of transfers might be attempted, since the progress at each stage can be readily calculated, with improved values of the partition ratios as they become available.

Example 5. Fig. 1 shows the distribution of an extract obtained from urine after a drug labelled with ^{14}C had been administered to animals. There are clearly mainly two substances present, although the experimental error is obviously considerable. The bad shape of zone A can be attributed to loss of an approximately constant quantity of material during the working up of each tube content for counting, and is not germane to the present inquiry. Analysis of the results was attempted in order to see if minor components could be detected. One seemed possible between the two main zones A and B. A two-component analysis of the zones A and B was first carried out with the p values nearest to the peaks, giving: A, 882 ± 48 counts/min.; B, 611 ± 49 counts/min.

With these values the calculated curve showed little evidence of material between the zones A and B, but most of the points on the extreme right of the figure lay beyond the calculated curve. (Tube 38 was, of course, omitted from the calculations.) This suggested the presence of a third substance, C, somewhere in this region. Trial analysis of a two-component mixture with tubes 32–45 gave the following values of R: $q = 0{\cdot}18$, $R = 255{\cdot}44$; $q = 0{\cdot}19$, $R = 265{\cdot}85$; $q = 0{\cdot}17$, $R = 249{\cdot}78$. Since the value of R was still decreasing as q changed from 0·19 to 0·17 a further trial at $q = 0{\cdot}16$ was carried out, giving $R = 260{\cdot}67$. The minimum for the fitted curve for values of p between 0·16 and 0·18 was close to $p = 0{\cdot}17$, which was taken as sufficiently accurate for the purpose. With this figure the values are: $B = 570 \pm 20$ counts/min.; $C = 93 \pm 21$ counts/min. Since C is more than four times its standard deviation there can be little doubt about it being a real substance. Its partition ratio is 4·8, a figure which gave a clue to its identity. Since it was thought that the presence of this third substance might affect the value of the other two, the analysis of the three-component system was carried out, giving: $A = 882 \pm 45$ counts/min.; $B = 574 \pm 47$ counts/min.; $C = 92 \pm 45$ counts/min. These figures were used to draw the fitted curve shown in Fig. 1.

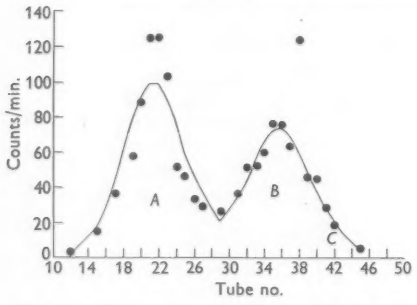

Fig. 1. Distribution of metabolites labelled with ^{14}C. 49 transfers. Continuous line is best fitted curve (see text, Example 5).

It is seen that the recalculation was hardly necessary. Provided that it is reasonably certain that the substances at the extremes in a zone do not overlap it is usually better to break up the analysis into parts where possible. It is also to be noted that C is now barely significant, due to the very large errors in A. It is probable, too, that here much the best estimate of the total amount of A is found from the tube at the peak, for the reason already stated. This is nearer 1200 counts/min. In erratic results of this kind some discrimination is necessary in drawing conclusions from the statistical calculations, although useful information can still be found.

DISCUSSION

Published methods for analysing a mixed zone in countercurrent distributions where no clear peaks can be distinguished have been mainly of a trial-and-error nature. Such methods can be greatly improved in reliability and accuracy by the use of suitable statistical procedures such as those presented here. The arithmetic involved may seem rather an undertaking for those whose main interest is in experiment, but would hold no terrors for a statistician with mechanical aids at his disposal, and would in many cases be fully justified by the information which can be obtained. If repeat analyses of mixtures of the same materials under identical conditions were being made a large proportion of the arithmetic need be done once only. Even with single substances it may happen that objective values with confidence limits for the amounts of substance and the partition ratios are desired, and these can be found by the 'least squares' method. If some idea is available of the likely experimental error a judgement can be made of the purity of the sample by the presence or absence of a systematic bias greater than would be expected by chance alone. Further, if such a bias is found, the amount and partition ratio of the

impurity can be calculated, although naturally with relatively small amounts of impurity the precision will not be great.

The regression method for the analysis of mixtures is quite general and could be applied to any series of properties of a mixture which were additive. Thus satisfactory results have been obtained in a similar manner with absorption spectra of mixtures. Further, the method should be applicable to series withdrawn from a countercurrent distribution, since for a given partition ratio the expected amounts in each fraction can be calculated, at least approximately (Karlson & Hecker, 1950; Compere & Ryland, 1954). Even if the mixture behaved in a non-additive but known manner, the method could still be applied, but this would involve a greater amount of numerical computation, which may not always be justified.

A method for analysing a binary mixture by distribution has been described by Morton & Tinley (1956) but is limited to pairs of substances with fairly large differences in partition ratios. The present method, although arithmetically more elaborate, can be used quite successfully with substances whose partition ratios differ by very little. It is only necessary to apply a sufficient number of transfers to get differences in values from those predicted for a single substance greater than the experimental error in a reasonable number of tubes. Further, the method is not limited to only two components.

The one major limitation is that the partition ratios should be independent of concentration, and of the presence of other substances. Little can be done with results such as those for oleic acid or the taurine-conjugated bile acids described by Ahrens & Craig (1952*a*, *b*).

SUMMARY

1. A 'least squares' method is described for calculating the amount of material in a zone containing a single substance observed after a countercurrent distribution. The calculation uses measurements on all relevant tubes. Partition ratios are assumed to be constant.

2. The method can be extended to give a 'best estimate' of the partition ratio of the substance.

3. The method can be extended to the calculation of the composition of zones formed by mixtures, and to estimates of the partition ratios of the components. So long as some measurable separation is achieved the method is applicable.

4. Examples are given of applications of the technique to actual experiments including some from the literature.

The author is greatly indebted to Dr O. L. Davies of this Department for help and advice, and for suggesting the method by means of which partition ratios can also be determined.

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The Purification and Properties of the L-Threonine Deaminase of the Rumen Micro-organism LC1

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The micro-organism LC, isolated from the sheep rumen by Elsden, Volcani, Gilchrist & Lewis (1956), ferments a variety of substrates to yield volatile fatty acids (Elsden & Lewis, 1953). Washed cells of the organism fermented L-threonine and L-serine. Hydrogen, carbon dioxide and ammonia were produced from both substrates and, in addition, propionate was formed from threonine and acetate from serine (Lewis & Elsden, 1955). Acetone-dried powders converted L-threonine into a keto acid, identified as 2-oxobutyrate, as well as the above end products. When 2-oxobutyrate was used as substrate, hydrogen, carbon dioxide and propionate were the principal products. These experiments suggested that a threonine deaminase is involved and that 2-oxobutyrate is an intermediate in threonine fermentation.

$$CH_3 \cdot CH(OH) \cdot CH(NH_2) \cdot CO_2H \rightarrow CH_3 \cdot CH_2 \cdot CO \cdot CO_2H + NH_3.$$

Similarly, pyruvate appears to be an intermediate in the breakdown of L-serine by this organism.

The deamination of threonine by both animal tissues and microbes has been studied by a number of workers in recent years, particularly with respect to cofactor requirements. The L-threonine deaminase of *Neurospora* has been completely resolved by Yanofsky & Reissig (1953) and shown to require pyridoxal phosphate for its action. Deaminases for both D- and L-threonine have been found in *Escherichia coli*, and Metzler & Snell (1952) have shown a pyridoxal phosphate requirement for the D-threonine deaminase. However, the situation with regard to the enzyme specific for the L-isomer in *E. coli* is more complicated. Wood & Gunsalus (1949) found that adenosine 5-phosphate (AMP) and glutathione (GSH) were necessary for the maximum activity of their partially purified enzyme. These results were not confirmed by Umbarger & Brown (1956), who studied crude extracts of *E. coli* and found no effect with AMP or GSH or both, but some increase in activity with pyridoxal phosphate. More recent studies by Umbarger & Brown (1957) indicate that two L-threonine deaminases may be present in *E. coli* extracts, one requiring AMP, GSH and pyridoxal phosphate for full activity [cf. the findings of Sayre & Greenberg (1956) with the sheep-liver enzyme], and the other requiring only pyridoxal phosphate.

This paper describes both the purification and properties of the L-threonine deaminase of LC and the almost complete resolution of the purified enzyme. A preliminary report of these findings has already been published (Walker, 1957).

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MATERIALS AND METHODS

Materials

Growth of the organism. LC strain 1 (LC1) of the rumen organism described by Elsden *et al.* (1956) was used throughout the present work.

Stock cultures (transferred fortnightly) were maintained on semi-solid medium containing 1·4% (w/v) of sodium lactate, 0·4% of Difco yeast extract, 0·02% of thioglycollic acid and 0·2% of agar in tap water, pH 7·4. Portions (10 ml.) in $\frac{5}{8}$ in. × 5 in. test tubes closed with cotton-wool plugs were sterilized by autoclaving at 15 lb./in.^{2} for 20 min. Oxygen was removed from the medium, immediately before inoculation, by heating at 100° for 10 min. and cooling. With this technique, a vigorously growing culture was obtained in 8–12 hr. All cultures were grown at 37°.

The method for large-scale cultivation of the organism was devised in this laboratory by Dr J. L. Peel (unpublished work) and was based on the methods used by Stadtman & Barker (1949) with *Clostridium kluyveri*. Commercial, 10 gal. iron drums, adequately cleaned, were used as culture vessels. The outlet at the top of the drum was provided with a rubber bung fitted with two glass tubes. One of these reached the bottom of the drum and was the inlet for gassing; the other, the gas outlet, terminated at the lower surface of the bung. This accessory was wrapped and sterilized separately. The drum was almost filled with tap water, closed with a cotton plug and sterilized by autoclaving for 2 hr. at 20 lb./in.^{2}.

The medium employed for large-scale cultivation contained 1% (w/v) of corn-steep liquor (a gift from the Distillers Co. Ltd., Wimbledon, Surrey), 1·4% of sodium lactate and 0·02% of sodium dithionite as reducing agent. The individual components of the medium were sterilized separately in concentrated solution and added aseptically to the water in the drum. Sodium lactate was sterilized as an approx. 35% solution by autoclaving at 15 lb./in.^{2} for 30 min. A 10% (w/v) solution of sodium dithionite was made up immediately before inoculation and sterilized by filtration.

Difficulty was experienced in sterilizing the corn-steep liquor, which contained a thermophilic rod-shaped organism resistant to autoclaving at neutral pH. The following procedure was therefore adopted: the corn-steep liquor, which is approx. N-acid, was diluted with an equal volume of water and autoclaved for 45 min. at 20 lb./in.^{2}. The solution was then neutralized to pH 7·4 with NaOH and filtered to remove precipitated material, after which it was autoclaved for 20 min. at 15 lb./in.^{2}. A further precipitate formed and was allowed to settle. The supernatant was poured into the drum aseptically and the precipitate discarded.

The inoculum was 2 l. of vigorously growing culture, grown in a 3 l. flask under an atmosphere of H_2–CO_2 (95:5). The arrangement for gassing was such that the culture could be expelled from the flask by reversing the direction of the flow of gas. The medium, which was the same as that used for drum-scale culture, was inoculated with a tube of cells growing vigorously in the semi-solid medium. After growth for 16 hr., the 2 l. of culture was transferred to the drum by displacement with the H_2–CO_2 gas mixture. The drum was flushed with the H_2–CO_2 mixture and incubated at 37°. Growth was complete in 12–16 hr. Considerable quantities of H_2 and CO_2 are produced during the growth of LC, and to avoid the possibility of an explosion the gases were led out of the confined space of the constant-temperature room via a water trap.

With the large inoculum used, strict asepsis was not obligatory; if the drum was well washed and filled with hot water from the tap, the autoclaving could be dispensed with. Similarly, the medium constituents could be added through a sterilized funnel without undue risk of contamination. When these procedures were adopted, however, the purity of the culture was checked by microscopic examination. This is a reasonably effective criterion of culture purity, as the morphology of the organism is highly characteristic (Elsden *et al.* 1956).

After growth the cultures were harvested with the Sharples Super centrifuge, washed once with freshly boiled, cooled water and dried *in vacuo* over H_2SO_4 at room temperature. The dried cells were pulverized and stored at −20°.

Reagents. The phosphate buffers used were prepared from solutions of M-KH_2PO_4 and M-Na_2HPO_4. All reagents used were of A.R. grade, except those listed below.

DL- and L-Threonine were obtained from L. Light and Co. Ltd. (Colnbrook, Bucks). The purity was checked by the periodate method of Shinn & Nicolet (1941) and the samples were found to contain 99% of 2-amino-3-hydroxy acid. No impurities were detected on chromatography in butanol–acetic acid–water (4:1:5) (upper phase mobile) and phenol–water (mutually saturated, the upper phase mobile). L-Serine was obtained from the Nutritional Biochemicals Corpn. (Cleveland, Ohio, U.S.A.). 2-Oxobutyric acid was prepared from 2-aminobutyric acid by the method of Keilin & Hartree (1936). The product was redistilled *in vacuo*, and the oily liquid so obtained stored at −20°. Solutions of the sodium salt were prepared by neutralizing solutions of the acid with approx. 2N-NaOH. When assayed with yeast carboxylase (Umbreit, Burris & Stauffer, 1949), the yield of CO_2 was theoretical. Crystalline sodium pyruvate was prepared by the method of Robertson (1942), except that the commercial pyruvic acid was first redistilled *in vacuo*. The final product gave a theoretical yield of CO_2 in the yeast carboxylase system. Pyridoxal phosphate was kindly supplied by Dr Morrison of Roche Products Ltd. (Welwyn Garden City, Herts). Calcium phosphate gel–cellulose columns were prepared by mixing calcium phosphate gel suspension (McIntire, Palmer & Marshall, 1945) with dry powdered cellulose to give a dry weight ratio of one part of calcium phosphate gel to five of cellulose. Glass columns 2 cm. in diameter were filled to a depth of about 12 cm. and the material was allowed to settle under gravity, finally giving a column about 8 cm. high.

Methods

Assay of enzymic activity. The unit of enzymic activity was defined as that amount of enzyme necessary to catalyse the formation of 1 μmole of 2-oxo acid under the standard conditions of assay. The conditions employed for assay were as follows: a sample of the enzyme preparation was incubated with 0·7 ml. of 0·1M-borate buffer, pH 9·5, 10 μg. of pyridoxal phosphate and 20 μmoles of L-threonine, in a total volume of 1 ml. The reaction was allowed to proceed for 5 min. at 37° and stopped by the addition of 0·2 ml. of 10N-H_2SO_4. After making the volume to 3 ml. the precipitated protein was centrifuged off and a sample of the supernatant assayed for keto acid by the extraction method of Friedemann & Haugen (1943).

The specific activity of an enzyme preparation was defined as units/mg. of protein.

Protein. This was determined by the method of Stadtman, Novelli & Lipmann (1951), on the assumption that 0·1 mg. of protein gives an optical density of 0·1 at 340 mμ under their conditions. This method was used whenever specific-activity determinations were made.

Ammonia. This was determined by the method of Conway & O'Malley (1942).

RESULTS

Table 1 summarizes the results obtained from the purification procedure adopted.

Extraction of the dried cells. Vacuum-dried cells of LC1 were suspended in 0·01M-phosphate buffer, pH 7·8 (1 g. of cells/10 ml. of buffer), and the suspension was stirred at 37° for 1 hr. with a magnetic stirrer. All subsequent operations were carried out below 5°. Cell debris was removed by centrifuging at −2° for 15 min. at 24 000 *g* in an International refrigerated centrifuge. The clear, yellow–green supernatant contained the deaminase activity.

Protamine sulphate treatment. The crude extract was treated with a solution containing 15 mg. of protamine sulphate/ml.; 1 ml. of this solution was added for each 100 mg. of extract protein and the mixture stirred for 15 min.; the precipitate was then removed by centrifuging and discarded.

Ammonium sulphate fractionation. Solid $(NH_4)_2SO_4$ was first added to 40% saturation, the amount required being calculated from the nomogram of Dixon (1953). The mixture was then stirred for 15 min. The precipitate was removed by centrifuging and discarded. Further addition of

Table 1. *Purification of* L-*threonine deaminase*

Purification stage	Total activity (units)	Total protein (mg.)	Specific activity (units/mg. of protein)
Crude extract from 3 g. of dried cells	1330	266	5·0
After treatment with protamine sulphate	1330	240	5·5
Pooled fractions from gradient elution with $(NH_4)_2SO_4$	1298	59·6	22·2
Individual fractions from calcium phosphate gel–cellulose column*	50	0·30	166
	31	0·06	516
	39	0·17	230
	60	0·49	122
	66	0·11	600
	56	0·11	508
	40	0·40	100
	23	0·17	135

* These fractions were not pooled. For reasons see text.

solid $(NH_4)_2SO_4$ was made until the solution was 65% saturated. After stirring for 15 min. the precipitate was centrifuged off and suspended in 70% saturated $(NH_4)_2SO_4$ solution.

The precipitated protein was then fractionated by concentration-gradient elution, the method of Schwimmer (1953) as modified by Sayre & Greenberg (1956) being used. The suspension of protein in 70% saturated $(NH_4)_2SO_4$ solution was mixed with Celite 535 (Johns-Manville Co., London) to form a thin slurry, which was then placed on the top of a column of Celite 535 (5 cm. diam. × 8 cm. high) made up in 70% saturated $(NH_4)_2SO_4$. The column was attached to a conical closed flask containing 300 ml. of 70% $(NH_4)_2SO_4$ stirred by a magnetic stirrer. The gradient was established by running in 0·05M-phosphate buffer, pH 7·8. Fractions of approx. 20 ml. were collected, the protein contents of which were followed by measuring the density at 280 mμ in the Unicam SP. 500 spectrophotometer. Protein with deaminase activity was eluted in a fairly sharp band with nearly constant specific activity.

Absorption and elution from calcium phosphate gel–cellulose column. The active fractions from the gradient elution column were combined and the protein was precipitated by adding solid $(NH_4)_2SO_4$ to saturation. The precipitate was removed by centrifuging, dissolved in a small volume of water and the solution placed on a calcium phosphate gel–cellulose column, which had been washed for 4–5 hr. with 0·01M-phosphate buffer, pH 7·8, containing 0·2% of NaCl (Swingle & Tiselius, 1951). Development was first carried out with 0·04M-phosphate buffer, pH 7·8, containing 0·2% of NaCl, and a yellow–green band of protein which had no deaminase activity was eluted. When the optical density (280 mμ) of the fractions had fallen to a constant low value (0·02), the eluting buffer was changed to 0·06M-phosphate buffer, pH 7·8, containing 0·2% of NaCl. Protein with deaminase activity was then eluted in a rather diffuse band. The specific activities of the fractions containing active protein were variable, probably because the method of estimating protein was not sufficiently sensitive with the small amounts of protein present (about 0·1 mg./5 ml. fraction). The specific activities of the fractions used in subsequent experiments averaged about 500 units/mg. of protein. Attempts to concentrate the small amounts of protein thus obtained by precipitation with $(NH_4)_2SO_4$ resulted in up to 75% loss of activity. For this reason, the untreated fractions were stored at −15°, where the activity did not fall off appreciably over 3–4 weeks.

The purification procedure was reproducible except that different batches of dried cells gave extracts of varying specific activity. This affected the degree of purification brought about by protamine sulphate treatment, which varied from 1·1- to 2·5-fold.

Except in a few preliminary experiments, the properties of the enzyme were studied with preparations obtained by the above purification procedure.

Properties of the enzyme

Lewis & Elsden (1955), using acetone-dried powers of LC3, studied the effect of pH on deamination of threonine and also isolated the deamination product as the 2:4-dinitrophenylhydrazine derivative. Repetition of these experiments with crude extracts of vacuum-dried cells showed that the optimum pH for 2-oxo acid production from threonine was over the range pH 9·0–10·0 (Fig. 1), whereas Lewis & Elsden (1955) found quite a sharp peak at pH 9·0. Over the range pH 7·0–10·0, the production of 2-oxo acid shows little sensitivity to changes in pH.

Isolation, as the 2:4-dinitrophenylhydrazone, of the 2-oxo acid produced from threonine by crude extracts of vacuum-dried cells confirmed the findings of Lewis & Elsden (1955) that the product is 2-oxobutyric acid. The product melted at 196° (uncorr.) and did not depress the melting point of an authentic sample of the 2:4-dinitrophenylhydrazone of 2-oxobutyric acid.

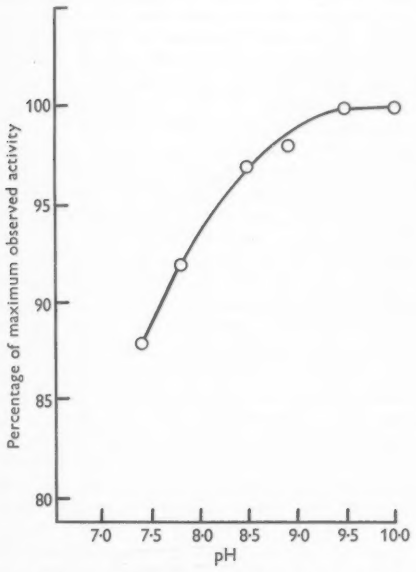

Fig. 1. Effect of pH on the initial rate of 2-oxo acid production from L-threonine by a crude extract of LC1. Reaction mixture was as described in the text. Phosphate buffer was used at pH 7·4 and 7·8 and borate buffer between pH 8·5 and 10·0. Rates were calculated from the portion of the progress curve where 2-oxo acid production was linear (0–10 min.).

With a crude extract of vacuum-dried cells, the production of 2-oxo acid and ammonia from threonine was followed. The two products appeared simultaneously and in equal amounts (Table 2).

Activity towards L-*serine.* Throughout the purification, threonine was always deaminated more rapidly than serine. The ratio of serine deaminase activity to threonine deaminase activity remained reasonably constant (Table 3). These results agree with those of Wood & Gunsalus (1949) and Yanofsky & Reissig (1953) with the L-threonine deaminase of *E. coli* and *Neurospora* respectively.

Effect of substrate concentration. An enzyme preparation purified approximately 50-fold (specific activity 340 units/mg. of protein) was used to study the effect of substrate concentration on the initial rate of deamination of L-serine and L-threonine. Data plotted by the method of Lineweaver & Burk (1934) gave K_m values of 6·8 mM and 3·0 mM for serine and threonine respectively.

Effect on D-*threonine.* DL-Threonine was deaminated at only 45% of the rate at which L-threonine was deaminated. In three fermentation

Table 2. *Equivalence of ammonia and 2-oxo acid formed*

Crude extract (0·1 ml.) was incubated with 0·5 ml. of 0·1 M-borate buffer, pH 9·5, in a stoppered test tube fitted with a side arm which contained 0·2 ml. of 0·1 M-L-threonine. The side-arm contents were tipped in after equilibration of the tubes at 37° and reaction was stopped at the time indicated by quickly adding 0·2 ml. of 10 N-H_2SO_4 via the stopper. Vessels were kept closed and shaken from time to time over 15 min. to ensure complete trapping of ammonia.

Incubation time (min.)	NH_3 formed (μmoles)	2-Oxo acid formed (μmoles)
0	0·0	0·1
5	3·7	3·9
10	6·8	6·9
15	8·7	9·0
20	9·4	9·5

Table 3. *Relative activities of deaminase preparations towards* L-*serine and* L-*threonine*

Enzyme preparation (0·1 ml.) was assayed in open tubes as described under Methods; L-serine (20 μmoles) was substituted for threonine where necessary. Keto acid production in the absence of substrate was negligible.

Stage of purification*	Specific activity of enzyme preparation (units/mg. of protein)	2-Oxo acid formed (μmoles) From L-threonine (*a*)	From L-serine (*b*)	Ratio (*a*/*b*)
Crude extract	2·1	8·6	2·6	3·3
After treatment with protamine sulphate	4·6	1·4	0·3	4·6
After gradient elution with $(NH_4)_2SO_4$	13·5	5·8	1·07	5·4
After calcium phosphate gel–cellulose column	290	0·82	0·15	5·5

* Enzyme preparations used in this experiment were selected at random from different batches at the various stages of purification.

experiments it was found that the yields of ammonia from DL-threonine were 60, 57 and 54% of the theoretical. These results indicate that the threonine deaminase of LC is inhibited by D-threonine and that the enzyme is specific for the L-isomer.

Inhibitors. Yanofsky & Reissig (1953) reported inhibition of the L-threonine deaminase of *Neurospora* by cyanide and hydroxylamine. A partially purified enzyme (specific activity 130 units/mg. of protein) from LC1 was inhibited 6 and 43% by 0·5 and 50 mM-KCN and 64% by 0·5 mM-hydroxylamine.

Cofactor requirement. In agreement with the findings of both Yanofsky & Reissig (1953) and Sayre & Greenberg (1956), no significant dissociation of the coenzyme–apoenzyme complex was observed during the purification procedure and no resolution was obtained on dialysis. Yanofsky & Reissig (1953) obtained a resolved preparation by storing fresh mycelium of *Neurospora* in the deep-freeze for several weeks. Vacuum-dried cells of LC1 stored in the same way for up to 4 years did not, on extraction, give enzyme preparations in which deaminase activity was significantly increased by the addition of pyridoxal phosphate. The deaminase activity of the extracts was comparable with that of newer batches of cells. However, with a preparation which had been made by treating a crude extract with $(NH_4)_2SO_4$ to 90% saturation, freeze-drying the precipitate and storing at $-20°$ for 9 months, the rate of formation of 2-oxo acid from threonine was nearly doubled by adding 100 μg. of pyridoxal phosphate to the reaction mixture. The effect of freeze-drying on other preparations was then studied.

A crude extract of vacuum-dried cells was freeze-dried without further treatment. Portions of the dry material were stored at $+4°$ and $-20°$ and tested periodically for stimulation of deaminase activity by pyridoxal phosphate. Over a period of several weeks, no stimulation of 2-oxo acid production was observed. Results with purified enzyme preparations were somewhat variable, but a marked resolution was accomplished. Freeze-drying the purified enzyme without previous additions resulted in the loss of most of the activity, which was not restored by adding pyridoxal phosphate or glutathione or both together, or by boiled crude extract. When glutathione was added to a purified preparation (specific activity 378 units/mg. of protein) to a final concentration of 4 mM and the solution freeze-dried, addition of 10 μg. of pyridoxal phosphate/ml. of reaction mixture doubled the deaminase activity of the enzyme so treated. A control sample of the enzyme preparation, freeze-dried without prior addition of GSH, was not stimulated by addition of pyridoxal phosphate. The experiments of Sayre & Greenberg (1956) showed that dilute solutions of their purified deaminase were protected from denaturation by $(NH_4)_2SO_4$. The effect of freeze-drying purified preparations in the presence of $(NH_4)_2SO_4$ was therefore studied, and some resolution was obtained when preparations were freeze-dried in the presence of either $(NH_4)_2SO_4$ or GSH. Greater resolution was obtained when both $(NH_4)_2SO_4$ and GSH were added before freeze-drying. The extent of dissociation of the coenzyme from the apoenzyme varied from preparation to preparation, but apparent resolutions [(increase in activity obtained by addition of pyridoxal phosphate/total activity in presence of pyridoxal phosphate) × 100] of 75 and 94% have been observed. The results of a number of freeze-drying experiments are given in Table 4.

Effect of other cofactors on reactivation of the apoenzyme. Wood & Gunsalus (1949) found that the enzyme from *E. coli* required both GSH and AMP. In order to study the effects of GSH and AMP on the enzyme from LC1 a purified preparation, partially resolved by freeze-drying in the presence of $(NH_4)_2SO_4$ alone, was used. Neither GSH nor

Table 4. *Effect of adding ammonium sulphate and glutathione before freeze-drying on the resolution of the enzyme*

Activity of the preparations was determined in the usual assay system (see Methods) in the presence and absence of 10 μg. of pyridoxal phosphate. Concentrations of $(NH_4)_2SO_4$ and GSH added before freeze-drying were 25% of saturation and 4 mM respectively.

Enzyme preparation (specific activity in parentheses)	Additions before freeze-drying		2-Oxo acid formed (μmoles)		Apparent resolution (%)
	$(NH_4)_2SO_4$	GSH	No addition (a)	+ Pyridoxal phosphate (b)	$\left(\frac{b-a}{b}\right) \times 100$
1. Crude extract (2·1)	−	−	1·21	1·26	4
2. Crude extract (1·8)	+	−	1·6	2·5	54
3. Purified (378)	−	−	0·4	0·4	0
4. Purified (378)	−	+	0·5	0·96	46
5. Purified (560)	+	+	1·36	3·8	64
6. Purified (508)	+	+	0·53	8·9	94
7. Purified (600)	+	+	0·23	0·85	75

AMP added together or separately increased the activity of the preparation, but addition of pyridoxal phosphate (final concentration 20 μM) doubled the activity. If pyridoxal phosphate and GSH were added together (final concentrations 20 μM and mM respectively), the activity was increased more than fivefold (Table 5). No explanation is offered for the inhibition by AMP of the pyridoxal activation, but it is interesting to note that Yanofsky & Reissig (1953) observed the same effect with the *Neurospora* enzyme.

Table 5. *Effect of glutathione, adenosine 5-phosphate and pyridoxal phosphate on a partially resolved enzyme preparation*

A 75%-resolved enzyme preparation (0·1 ml.) was incubated at 37° for 20 min. with 0·5 ml. of 0·1 M-borate buffer, pH 9·5, 0·2 ml. of 0·1 M-L-threonine, with GSH (1 μmole), AMP (1 μmole) and pyridoxal phosphate (10 μg.) added where indicated.

Expt. no.	Additions: GSH	AMP	Pyridoxal phosphate	2-Oxo acid formed (μmole)
1	−	−	−	0·06
2	+	−	−	0·06
3	−	+	−	0·06
4	−	−	+	0·13
5	+	−	+	0·33
6	+	+	−	0·06
7	−	+	+	0·06
Control (no substrate)	+	−	+	0

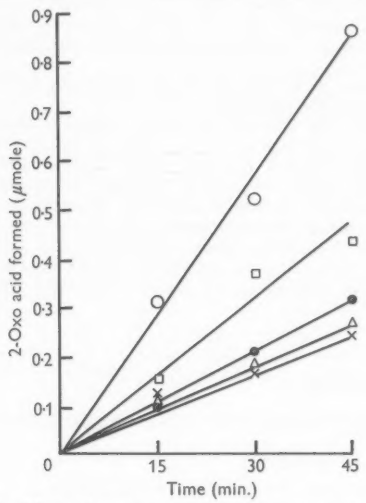

Fig. 2. Effect of pyridoxal phosphate concentration on production of 2-oxo acid by a partially resolved enzyme preparation. Reaction mixture contained 0·5 ml. of 0·1 M-borate buffer, pH 9·5, 0·2 ml. of 0·1 M-L-threonine, 0·1 ml. of 0·01 M-GSH and 0·1 ml. of partially resolved enzyme preparation. Pyridoxal phosphate was added to the concentrations indicated. Total volume 1·2 ml. Pyridoxal phosphate concentrations: ×, none; △, 0·2 μM; ●, 0·4 μM; □, 4 μM; ○, 400 μM.

Effect of pyridoxal phosphate concentration. An enzyme preparation with an apparent resolution of 75% (preparation 7, Table 4) was used to study the effect of pyridoxal phosphate concentration in the presence of 10 mM-GSH, and excess of substrate (Fig. 2). The values for initial rate of keto acid production have been corrected by subtraction of the value obtained in absence of added pyridoxal phosphate, and the velocities so obtained used to obtain a rough measure of the K_m value by the method of Lineweaver & Burk (1934). This treatment is valid if it is assumed that the added pyridoxal phosphate does not influence the activity of the undissociated enzyme–coenzyme complex. The K_m obtained (1·8 μM) represents the apparent dissociation constant of the pyridoxal phosphate from the apoenzyme.

DISCUSSION

Umbarger & Brown (1957) showed that when crude extracts of *E. coli* were treated with hydroxylamine according to the procedure described by Roberts (1952), the threonine deaminase was inactivated and that preparations so treated were reactivated by pyridoxal phosphate. Yanofsky & Reissig (1953), using purified preparations of *Neurospora* L-threonine deaminase, and Sayre & Greenberg (1956) using the sheep-liver enzyme, have also shown a requirement for pyridoxal phosphate.

The experiments reported here provide the first clear-cut evidence for a pyridoxal phosphate requirement by a purified, bacterial L-threonine deaminase.

The method employed for the dissociation of the apoenzyme–coenzyme complex is empirical; it was based on the finding that a crude extract treated similarly, and for another purpose, was partially resolved with respect to pyridoxal phosphate. No explanation can, as yet, be offered for the function of ammonium sulphate. The most obvious explanation of the role of GSH is that it protects essential sulphydryl groups.

The K_m value obtained for the pyridoxal phosphate–apoenzyme system is probably not a true indication of the affinity of the apoenzyme for its coenzyme, since the latter is obviously tightly bound to its protein, and the Michaelis–Menten theory assumes a freely dissociable complex. However, the value obtained (1·8 μM-pyridoxal phosphate) serves to characterize the preparation, and is in reasonable agreement with that of 0·4 μM given by Yanofsky & Reissig (1953) for the same system in *Neurospora*.

Optimum deamination of threonine proceeds over the range pH 9·0–10·0. This includes the optimum pH of 9·3 for the *Neurospora* enzyme (Yanofsky & Reissig, 1953). Sayre & Greenberg (1956) reported that the optimum pH for the sheep-liver enzyme was 7·4.

L-Serine appears to be deaminated in LC1 by the same enzyme which acts upon L-threonine, a finding in agreement with the work by Umbarger & Brown (1957) on the *E. coli* enzymes and Yanofsky & Reissig (1953) with *Neurospora*. In contrast, Sayre & Greenberg (1956) demonstrated that two easily separated enzymes were present in sheep liver: one specific for L-serine and the other for L-threonine.

The K_m values for L-threonine and L-serine were 3·0 and 6·8 mM respectively. These figures are similar to those found by Yanofsky & Reissig (1953) for the *Neurospora* enzyme, and by Wood & Gunsalus (1949) for the deamination of L-threonine by *E. coli* extracts. The sheep-liver enzyme has a lower affinity for its substrate ($K_m = 29$ mM; Sayre & Greenberg, 1956).

SUMMARY

1. The L-threonine deaminase of the rumen micro-organism LC1 was purified over 100-fold from extracts of vacuum-dried cells by successive fractionation with protamine sulphate and ammonium sulphate, gradient elution with ammonium sulphate and fractionation on a calcium phosphate gel–cellulose column.

2. The enzyme has an optimum pH in the range 9·0–10·0. K_m values of 6·8 mM and 3·0 mM were obtained for L-serine and L-threonine respectively. D-Threonine appeared to inhibit the enzyme.

3. The ratio of L-serine deaminase activity to L-threonine deaminase activity varied little at different stages of the purification, suggesting that one enzyme attacks both substrates.

4. The purified enzyme, after freeze-drying in the presence of ammonium sulphate or glutathione, required pyridoxal phosphate. Glutathione also appeared to be necessary for maximum activity.

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Condensed Tannins

1. A STUDY OF COMPLEX LEUCO-ANTHOCYANINS PRESENT IN CONDENSED TANNINS

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Condensed tannins are widely distributed in Nature, and constitute a heterogeneous group possessing the common characteristic of forming insoluble 'phlobaphenes' on treatment with hot dilute mineral acids in aqueous medium. This rather general characteristic is shared by the catechins, which occur in high proportion in association with tannins in some commercial tanning extracts, e.g. cube gambier from the leaves of *Uncaria gambir* and cutch from the heartwood of *Acacia catechu*. Freudenberg & Maitland (1934) and Freudenberg & Weinges (1955)

considered the highly complex condensed tannins to be polymers of catechins, a view also supported by Hathway & Seakins (1957). So far it has not been possible, however, to obtain direct structural evidence of the C_6–C_3–C_6 nature of the hypothetical basic units present in naturally occurring condensed tannins, although it is readily conceded that the evidence provided specifically for gambier and cutch extracts appears to be conclusive.

The remaining noteworthy concept is the leuco-anthocyanin hypothesis of Bate-Smith & Swain (1953), who regarded leuco-anthocyanins as well as catechins as possible prototypes of condensed tannins, from a study of the absorption spectra and phlobaphene reaction of these substances. Plant extracts which are classed as 'condensed tannins' and widely used for commercial tannage were shown by Pigman, Anderson, Fischer, Buchanan & Browning (1953) to be rich in leuco-anthocyanins. The extracts examined include those from black-wattle bark (*Acacia mollissima*), quebracho heartwood (*Schinopsis balansae* and *S. quebracho-colorado*), mangrove bark (mainly *Rhizophora* spp.), western hemlock wood (*Tsuga heterophylla*) and the bark of the black spruce (*Picea mariana*). The scarlet colorations developed from black-wattle and quebracho extracts were shown to exhibit lower absorption maxima (532 and 525 mμ respectively) than those obtained from other extracts (540–545 mμ). Hillis (1956*a*, *b*) found that 'condensed tannins' suitable for tanning purposes, and rich in leuco-anthocyanins, are also located in the heartwoods of various *Eucalyptus* species and in the bark of *Pinus radiata*.

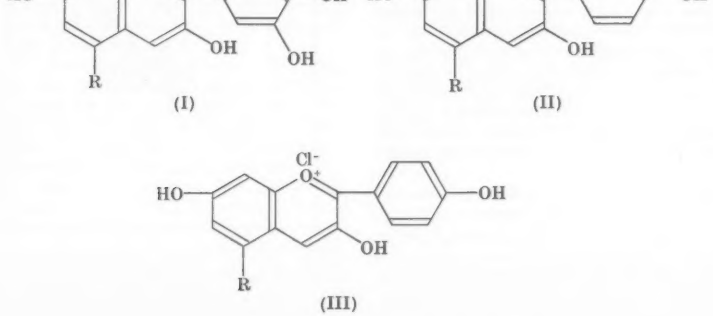
King & Bottomley (1954) and King & Clark-Lewis (1955) linked the structure of the monomeric leuco-anthocyanin, melacacidin, with that of the condensed tannins. They, however, overlooked the fact that monomeric flavan-3:4-diols are incapable of tanning collagen, and provided no evidence of the presence of complex leuco-anthocyanins in those condensed tannins used for commercial tannage.

The present work shows that both wattle and quebracho extracts contain appreciable proportions of monomeric and polymeric leuco-anthocyanins of the same C_6–C_3–C_6 structural pattern, and with varying affinity for cellulose and collagen in aqueous medium. The flavan-3:4-diol structure thus appears to form the basis for at least one type of condensed tannin, i.e. that based on leuco-anthocyanins. Anthocyanidins resulting from wattle and quebracho extracts belong to a new group compared with the naturally occurring delphinidin, cyanidin and pelargonidin series studied by Bate-Smith (1954). This has necessitated a more detailed study of chromatographic identification methods for anthocyanidins and for simple and complex leuco-anthocyanins (Roux, 1957*a*, *b*), as well as a detailed study of the molecular-weight distribution of wattle and quebracho tannins.

EXPERIMENTAL

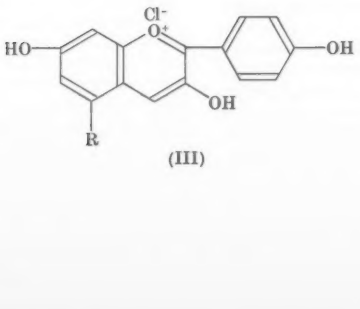
Synthesis and origin of anthocyanidins. Robinetinidin chloride or 3:7:3′:4′:5′-pentahydroxyflavylium chloride (I, R = H), fisetinidin chloride or 3:7:3′:4′-tetrahydroxyflavylium chloride (II, R = H) and 3:7:4′-trihydroxyflavylium chloride (III, R = H) were synthesized as previously described (Roux, 1957*a*).

Other anthocyanidins were obtained from natural sources. 3:7:8:3′:4′-Pentahydroxyflavylium chloride was obtained by generation from melacacidin (King & Bottomley, 1954), separated from the heartwood extractives of *Acacia melanoxylon* by using the 'preparative' method. Water containing 2% of acetic acid was used as irrigant for thick cellulose sheets (Whatman no. 3 or 3MM) and the



(I)

(II)



(III)

melacacidin was eluted with 70% ethanol after chromatography. Contaminants were removed by repeating the process with butanol–acetic acid–water (4:1:5, upper phase) as irrigant. The anthocyanidin was generated from melacacidin by the method of Pigman *et al.* (1953).

Anthocyanidins containing the phloroglucinol nucleus were generated from naturally occurring anthocyanins present in flowers by hydrolysis with 3N-HCl on the water bath. The aqueous solutions were extracted with amyl alcohol to furnish a concentrate of the anthocyanidin (Bate-Smith, 1954). Delphinidin (I, R = OH) was obtained from *Delphinium* and *Salvia* spp.; cyanidin (II, R = OH) from the rose Daily Mail (scented), and pelargonidin (III, R = OH) from various *Pelargonium* spp.

Chromatographic identification of anthocyanidins. Chromatograms were run on Whatman no. 1 paper in two solvent systems: (*a*) the 'Forestal Solvent', namely water–acetic acid–conc. HCl (10:30:3, v/v) and (*b*) 90% (w/v) formic acid–3N-HCl (1:1, v/v) at 21°. After noting the R_F values the absorption maxima of the anthocyanidins were determined directly on the paper by the method of Bradfield & Flood (1953). Finally, after allowing the escape of excess of HCl from the paper by airing for 4–7 days at room temperature (20°), the anthocyanidin spots were treated with a few drops of 5% aluminium sulphate solution. The paper was allowed to dry in air and the absorption maximum of the blue colorations developed were determined in the spectrophotometer as before. Visual comparison and distinction between the various shades of blue could be made by comparison with reference compounds.

The above procedures are complicated by the presence of 'background' polyphenols, particularly where anthocyanidins are generated from condensed tannin mixtures. In such instances the pigment generated was applied in an intense streak on the origin of the paper chromatogram. After development, preferably in 90% formic acid–3N-HCl mixture, which tends to keep the highly condensed tannins at the origin, the scarlet band was cut out as a strip. The strip was cut and developed on the wick principle (Reith, 1957) with methanol. Tannins and other phenolic compounds tend to move forward while the anthocyanidins lag behind. The strip is removed once the methanol front nears the top of the tube and the paper is air-dried. The absorption spectrum of the anthocyanidin is investigated as above with the spectrophotometer. The trailing portion of the scarlet coloration was found to give the most satisfactory measurements of absorption maxima where other contaminants are present.

Generation of anthocyanidins from leuco-anthocyanins. The method of Pigman *et al.* (1953) was used and the scarlet colorations developed were examined qualitatively by means of paper chromatography, or used for estimating the yield of anthocyanidin quantitatively. Where the rate of generation of anthocyanidins from leuco-anthocyanins or the stability of the anthocyanidins were studied a number of similar tubes were used, and these were removed from the boiling-water bath at regular intervals and cooled quickly.

Origin and synthesis of monomeric leuco-anthocyanins or flavan-3:4-diols. Melacacidin, 7:8:3′:4′-tetrahydroxyflavan-3:4-diol (King & Bottomley, 1954) was obtained from the methanolic extract of the heartwood of *Acacia melanoxylon* by the preparative method with chromatographic sheets, as previously outlined.

7:3′:4′:5′-Tetrahydroxyflavan-3:4-diol, leucorobinetinidin, and 7:3′:4′-trihydroxyflavan-3:4-diol, leucofisetinidin, were obtained by the catalytic hydrogenation of dihydrorobinetin and dihydrofisetin (fustin) respectively with platinum oxide as catalyst by the method of Freudenberg & Roux (1954).

5:7:3′:4′-Tetrahydroxyflavan-3:4-diol, leucocyanidin, was obtained by the reduction of dihydroquercetin (taxifolin) with sodium borohydride by the method of Swain (1954).

Identification of leuco-anthocyanins on paper chromatograms. Toluene-*p*-sulphonic acid (3–5%, w/v) in absolute ethanol is evenly sprayed on two-way paper chromatograms. After heating the paper at 80–90° in an air oven, red spots developed in those areas in which leuco-anthocyanins are present. During the heating period (5–10 min.) the development of the pink area must be watched, as prolonged heating causes the cellulose to darken, obscuring the colours which have developed. The sheets may be rolled into a tube and heated in an oven, provided that the chromatographic paper rests on thick absorbent paper and remains out of contact with the hot metal frame of the oven. Where this procedure is adopted the paper must be removed at 5 min. intervals for inspection. The paper is quite stable after development of the spots and may be stored for record purposes.

Fractionation of black-wattle tannins for study of leuco-anthocyanins. Black-wattle tannins, when examined by two-way paper chromatography, may be arbitrarily subdivided into a more mobile fraction which travels mainly as discrete spots *A*–*F*, and a low R_F trail in which no clear differentiation of constituents is apparent (Fig. 1).

Constituents of the mobile higher R_F fraction were separated on a micro-scale by paper chromatography on thick cellulose sheets (Whatman no. 3 or 3MM, 18½ in × 22½ in) by the 'preparative' method, as outlined by Nordström & Swain (1953) and others. Fresh wattle bark or commercial extract were exhaustively extracted with methanol in order to eliminate the gums naturally present. The methanolic solution was streaked on to the sheets and the chromatogram developed in butan-1-ol–acetic acid–water (6:1:2). By using traces of the brilliantly fluorescing flavanols naturally present (e.g. robinetin, R_F 0·51, and

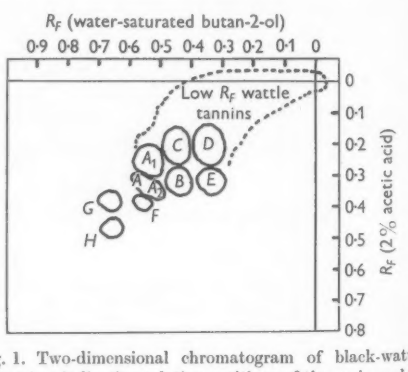


Fig. 1. Two-dimensional chromatogram of black-wattle tannins, indicating relative positions of the main mobile 'constituents' and the low R_F trail.

Table 1. *Preliminary fractionation of black-wattle tannins*

Fraction	Extraction period	Polyphenolic tannins* (%)	Percentage of total polyphenols	Mol. wt.
C_1	24 hr.	82·6	49·5	1290
C_2	72 hr.	81·9	13·3	1305
C_3	24 hr.	45·5	26·2	1507
C_4	8 days	39·8	5·3	1556
C_5	14 days	38·7	3·2	1540

* Estimation by the ultraviolet method (Roux, 1951).

fisetin, R_F 0·72) as reference compounds, bands corresponding to constituents *D* and *E*, *B* and *C*, *A*, A_1, A_2 and *F*, and *G* and *H* were cut. The various groups of constituents were eluted with ethanol–water mixtures as described by Nordström & Swain (1953).

The constituents were separated by re-running the bands on paper sheets with 2% acetic acid as developing medium. The constituents were eluted as before and concentrated. Overlap of constituents occurred to a large extent, but by re-running the bands and by cutting and discarding strips between bands, good separation was achieved and the 'constituents', *A*, A_1, *B*, *C*, *D*, *E*, *G* and *H* were isolated in a form sufficiently 'pure' for their migration as discrete spots on two-way paper chromatograms when the above solvents were used. These constituents were, in fact, still not completely pure, as shown by further chromatography with water-saturated phenol as irrigant, and it must be assumed that overlap of constituents occurs even after using two solvent systems. The fractions or 'constituents' obtained by these methods were, however, sufficiently 'pure' for a study of their leuco-anthocyanin reactions.

Estimation of molecular weight of wattle and quebracho tannins

Materials. Owing to the complexity of the mixture wattle tannins were fractionated first by successive solvent extraction of the fresh mature bark, initially with ethyl acetate and finally with methanol (Table 1). Fractions C_1 and C_2 were obtained by successive extractions of macerated fresh bark with ethyl acetate, followed by three successive extractions with methanol, giving fractions C_3, C_4 and C_5. Fractions C_1 and C_2 are similar in tannin:non-tannin ratio and in molecular weight (1300), as were also fractions C_3, C_4 and C_5 (1500–1550). Fractions C_1 and C_3 were selected as representative fractions for detailed examination.

Quebracho extract ('ordinary' or 'unsulphited') from commercial sources (71·6% tannin content) was used without preliminary separation.

Chromatographic fractionation of tannins. The polyphenolic tannins were fractionated by paper chromatography on thick sheets, e.g. Whatman no. 3 or 3MM by the methods previously described (Nordström & Swain, 1953), with 2% acetic acid as irrigant. The presence of acetic acid limits atmospheric oxidation during paper chromatography After air-drying, strips were cut from the sheet corresponding to R_F values 0, 0·1, 0·2, 0·3, 0·4 and 0·5 + 0·6, so that the centre of each band corresponds to these R_F values. The bands (approx. 2 in. wide) were eluted with ethanol–water mixtures as previously described, and the eluent from each band concentrated under vacuum.

Sugars present in the extract migrate just behind the solvent front in the chromatographic separation and are therefore eliminated.

Preparation of methoxyl derivatives of tannins. The fractions were methylated with excess of diazomethane (8 g. of nitrosomethylurea to 0·5 g. of tannins) in methanolic solution maintained at −10° for 48 hr. Diazomethane reacts selectively with polyphenolic tannins and permits separation of any residual carbohydrate material during recovery of the product by pouring into water (Evelyn, 1954*a*). Substituted tannins were vacuum-dried at 110° over P_2O_5.

Analysis for methoxyl content. Methoxyl values of the fractions were determined by duplicate semi-micro Zeisel analysis, the bromine–acetate absorbent of Vieboch & Brecher (1930) being used.

Estimations of molecular weights. Molecular weights of the methylated tannins were determined in benzene with the Ray (1952) ebulliometer, as modified by Evelyn (1954*b*) and incorporating improvements detailed by Smith (1956). A further modification of the same apparatus (Evelyn, van Berge & Cooper, 1958) was used to determine molecular weights in chloroform or acetone for those methylated tannin fractions which are only partly soluble in benzene.

The apparatus constant was determined daily and the average value used for calculation of the molecular weights. Average molecular weights were obtained from the mean of readings at four concentrations, as the readings were independent of concentration.

Molecular-weight values of tannins. Results are given as the molecular weights of the unsubstituted polyphenolic tannins, and are calculated from the determined molecular weights of the methylated derivatives and their methoxyl values.

RESULTS

Identification of anthocyanidins

The R_F values of the anthocyanidins in the different solvent systems have been tabulated (Roux, 1957*a*). 3:7:4′-Trihydroxyflavylium chloride was erroneously represented as having R_F 0 in 90% formic acid–3N-HCl (1:1) mixture, owing to the presence of impurities resulting from synthesis. Subsequent work has shown the correct value to be R_F 0·6, and the anthocyanidin exhibits yellowish fluorescence under ultraviolet light. The absorption maxima of the anthocyanidins and their aluminium complexes (Roux, 1957*b*) provide additional parameters for their identification. The absorption

maxima of the aluminium complexes tend to be broad, and visual comparison with reference compounds provides useful confirmation of identity.

Differentiation between monomeric and polymeric leuco-anthocyanins

The flavan-3:4-diols were examined by two-way paper chromatography with butanol–acetic acid–water (4:1:5, upper phase) for direction 1 and 2% acetic acid for direction 2. The compounds were located on the chromatograms with the toluene-*p*-sulphonic acid reagent, and R_F values were noted (Table 2). King & Bottomley's (1954) melacacidin was found to consist of two compounds running to different R_F values in each solvent system. The shade of red developed with the reagent on heating varied for the different leuco-anthocyanins. Leucofisetinidin (7:3′:4′-trihydroxyflavan-3:4-diol) developed a purple shade initially which became light pink, whereas the melacacidins developed a deep red. The others formed red colorations of intermediate shade.

These synthetic and naturally occurring flavan-3:4-diols have a higher R_F value in 2% acetic acid or in water than catechins and 2:3-dihydroflavonols (Table 2), and are also more highly mobile than the polymeric tannins. Two-dimensional chromatograms run under the above conditions may therefore be used to locate monomeric flavan-3:4-diols (R_F 0·47–0·55 in 2% acetic acid) and also polymeric leuco-anthocyanins (R_F 0–0·45 in 2% acetic acid) with the aid of the toluene-*p*-sulphonic acid reagent.

Of 144 'heartwoods' examined from trees generally classified as 'hard-woods' (mainly those indigenous to South Africa, but also including exotic types), 44 (33%) gave strongly positive leuco-anthocyanin reactions, and 17 (12%) showed chromatographic evidence of the presence of monomeric forms (Table 3). The monomeric leuco-anthocyanins are usually accompanied by polymeric leuco-anthocyanins of lower R_F in 2% acetic acid. These were shown up prominently by the toluene-*p*-sulphonic acid reagent. In most species examined only polymeric leuco-anthocyanins are present, as judged by their low R_F on paper chromatograms and the formation of anthocyanidins under the conditions of Pigman *et al.* (1953).

Table 2. *R_F values of flavan-3:4-diols, catechins and 2:3-dihydroflavonols*

	R_F in developing solvent	
	Butan-1-ol–acetic acid–water	2% Acetic acid
Flavan-3:4-diols		
7:3′:4′-Trihydroxy-	0·76	0·49
7:3′:4′:5′-Tetrahydroxy-	0·57	0·55
7:8:3′:4′-Tetrahydroxy-	0·42	0·47
	0·50	0·54
5:7:3′:4′-Tetrahydroxy-	0·52	0·53
Catechins		
(+)-Gallocatechin	0·57	0·33
(+)-Catechin	0·76	0·39
(±)-*epi*-3:7:3′:4′-Tetrahydroxyflavan	0·92	0·42
		0·47
2:3-Dihydroflavonols		
Dihydrofisetin (fustin)	0·90	0·35
Dihydrorobinetin	0·74	0·36
Dihydroquercetin (taxifolin)	0·90	0·29
Dihydromyricetin (ampeloptin)	0·70	0·35

Table 3. *Leuco-anthocyanins in the 'heartwoods' of dicotyledonous plants*

All heartwoods listed give a strong reaction for leuco-anthocyanins; those marked * also contain monomeric leuco-anthocyanins.

Anacardiaceae	*Harpephyllum caffrum, Rhus legati,* Schinopsis quebracho-colorado** (syn. *S. lorentzii*), *S. balansae**
Celastraceae	*Gymnosporia acuminata, Pleurostylia capensis, Pterocelastrus tricuspidatus**
Cornaceae	*Curtisia faginea*
Cunoniaceae	*Cunonia capensis*
Ebenaceae	*Diospyros mespiliformis, Euclea lanceolata**
Euphorbiaceae	*Phyllanthus discoideus*
Flacourtiaceae	*Kiggelaria africana, Scolopia munduti*
Leguminosae	
Caesalpinioideae:	*Baikiaea minor, B. plurijuga, Brachystegia apendulata,* B. spiciformis, Burkea africana, Copaifera coleosperma,* C. tessmanii,* Erythrophleum africana, E. guineense, Isoberlinia tomentosa, Schotia latifolia**
Mimosoideae:	*Acacia dealbata,* A. decurrens,* A. melanoxylon,* A. mollissima,* A. nigrescens,* A. pycnantha**
Papilionatae:	*Berlinia niambiensis*
Myristicaceae	*Staudtia gabonensis*
Myrtaceae	*Eucalyptus marginata, Syzigium cordatum*
Ochnaceae	*Ochna arborea, Ochna* sp.*
Rhamnaceae	*Rhamnus zeyheri**
Rosaceae	*Pygeum africanum*
Rhizophoraceae	*Weihea africana*
Sapotaceae	*Mimusops obovata, Manilkara cuniefolia*
Ulmaceae	*Trema guineensis*

Generation of anthocyanidins from wattle and quebracho extracts

Quebracho tannins. Quebracho extract yields fisetinidin (mainly) and also a second anthocyanidin (traces) when heated with HCl in propan-2-ol solution under pressure (Roux, 1957*a*). On paper chromatograms the whole of the lower R_F area containing the tannins gives the leuco-anthocyanin reaction with toluene-*p*-sulphonic acid spray reagent, but the presence of other constituents, probably catechins, is indicated as brown spots (Fig. 2). This behaviour indicates the presence of leuco-anthocyanins throughout the quebracho-tannin fractions. On the paper chromatogram the leuco-anthocyanins overlap each other so as to form a continuous band. This streaking without separation into discrete spots may result from the complexity of the mixture and also from the structural complexity of the individual tannins. Brown constituents which remain on the starting-line in direction 2 do not react readily with the toluene-*p*-sulphonic acid spray, but nevertheless yield fisetinidin on treatment with HCl in propan-2-ol solution under pressure.

No pink coloration developed in that position (Fig. 2) where the monomeric leuco-fisetinidin, 7:3′:4′-trihydroxyflavan-3:4-diol, should be located. The absence of Freudenberg & Maitland's (1934) 'quebracho catechin', or (±)-*epi*-7:3′:4′trihydroxyflavan-3-ol, from commercial quebracho extract has also been established (Roux, 1957*b*).

Black-wattle tannins. Black-wattle extracts forms three pigments on treatment with HCl under the conditions of Pigman *et al.* (1953). Two of these have been identified as the anthocyanidins robinetinidin (major proportion) and fisetinidin, but the identity of the third pigment is unknown (Roux, 1957*b*). Robinetinidin is the only anthocyanidin generated from constituents *C* and *E*, whereas both robinetinidin and fisetinidin are generated from A_1. Constituents *B* and *D* give mainly the unidentified orange–yellow coloration. The anthocyanidins and the unknown pigment are also generated in about the same proportions as from the whole extract, from that fraction of wattle tannins which remains immobile on the starting-line in direction 2 (Roux, 1957*b*).

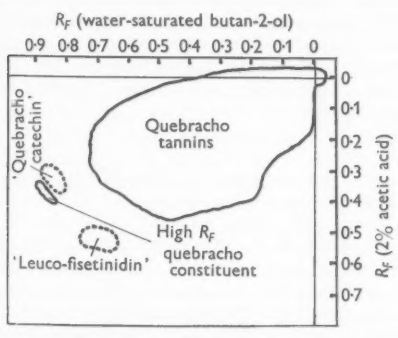


Fig. 2. Two-dimensional chromatogram of quebracho tannins, indicating relative positions of epi-7′:3′:4′-trihydroxyflavan-3-ol ('quebracho catechin') and 7:3′:4′-trihydroxyflavan-3:4-diol ('leuco-fisetinidin'). See text.

Yields of anthocyanidins. The amount of anthocyanidin generated from simple or complex leuco-anthocyanins increases rapidly over the first hour, but apparently the rate of reaction declines rapidly after this period. Robinetinidin and fisetinidin also undergo partial transformation into the corresponding flavonols, resulting in a decrease in the absorption density at the absorption maximum of each coloration, and other variables (Pigman *et al.* 1953) also affect the yield of anthocyanidins from leuco-compounds.

To obtain an indication of the extent to which leuco-anthocyanins occur in wattle and quebracho extracts, a comparative study of the yields of anthocyanidins from monomeric and complex leuco-anthocyanins of similar type was made. Thus most of the above variables are eliminated.

Quebracho extract yields mainly fisetinidin ($\lambda_{max.} = 525$ mμ), but for black-wattle extract three pigments are generated, ranging from 470 to 532 mμ in absorption maxima. Measurement of the percentage conversion for quebracho (at 525 mμ) and for wattle (at 532 mμ), as represented in Table 4, is limited by these factors, but nevertheless furnishes approximate values. Polyphenols and extracts were treated with HCl for 2 hr. under the conditions of Pigman *et al.* The percentage conversion is calculated from the absorption-density values of synthetic robinetinidin ($C_{15}H_{11}O_6Cl$, absorption density = 0·810 at 532 mμ and 3·5 mg./500 ml.), and fisetinidin ($C_{15}H_{11}O_5Cl,H_2O$, absorption density = 0·595 at 525 mμ and 2·3 mg./500 ml.). Correction is made during calculation of yields for the water content of fisetinidin.

Range of molecular weights in condensed tannins

Estimation of molecular weights of tannin fractions. The molecular weights of the extracts and of their fractions of different R_F values, as well as their weight distribution and percentage recovery, are given in Table 5. Wattle extracts C_1 and C_3 were obtained from the same bark (Table 1). No attempts were made to elute the strips exhaustively, as oxidation effects are likely to result from prolonged elution. The recovery of simple polyphenols, e.g. catechins, from paper chromatograms is, for example, never quantitative, in spite of their

Table 4. *Yield of anthocyanidins from tannins and flavan-3:4-diols*

Material	Weight (mg.)	Absorption density at 525 or 532 mμ	Conversion* into fisetinidin or robinetinidin (%)
Commercial quebracho	2·9	0·320	5·6†
7:3′:4′-Trihydroxyflavan-3:4-diol	6·7	0·438‡	23·8
Commercial wattle	3·7	0·366	6·3†
7:3′:4′:5′-Tetrahydroxyflavan-3:4-diol	2·9	0·201‡	26·9
Wattle fraction 4*A* 1 ('constituent' *E*)	2·2	0·169	3·0
Wattle fraction 3*A* 1 ('constituent' *C*)	2·9	0·378	5·1
Wattle fraction 2*A* 1 ('constituent' *A* 1)	2·6	0·296	4·4

* Conversions for quebracho and leuco-fisetinidin calculated as for fisetinidin, others as for robinetinidin.
† Calculated to a 'pure' tannin basis.
‡ Diluted to 500 ml., others to 50 ml.

Table 5. *Molecular-weight distribution of wattle and quebracho tannins*

	Wattle fraction C_1		Wattle fraction C_3		Quebracho extract	
Mean R_F	Tannins eluted (%)	Mol.wt.	Tannins eluted (%)	Mol.wt.	Tannins eluted (%)	Mol.wt.
0′	—	—	24·3	3240	—	—
0	3·7	1442	15·7	1631	21·0	2350
0·1	20·3	1287	24·3	1606	17·1	1811
0·2	24·3	1033	22·1	1203	16·5	1362
0·3	29·7	908	13·6	933	19·7	1066
0·4	15·6	649	—	—	17·0	910
0·5+0·6	6·5	554	—	—	8·7	790
Recovery (%)	71·0	—	74·0	—	95·0	—
Calc. av.mol.wt.	—	971	—	1826	—	1461
Determined av.mol.wt.	—	1284	—	1507	—	1327

Table 6. *Percentage yield of anthocyanidins from wattle- and quebracho-tannin fractions*

Mean R_F of fraction	Wattle fraction C_1	Wattle fraction C_3	Quebracho tannins
0′	—	4·39	—
0·0	4·33	4·96	3·36
0·1	5·78	4·50	3·56
0·2	4·03	5·09	4·00
0·3	4·34	3·89	4·53
0·4	3·63	—	4·08
0·5+0·6	2·27	—	3·34

relatively low affinity for cellulose. Only small quantities of residual tannins remained on the strips after elution with ethanol–water mixtures, with the exception of R_F 0 of wattle-fraction C_3. This band was subsequently eluted with acetone–water (1:1) to give fraction *O′*.

Generation of anthocyanidins from tannin fractions. Examination by two-way paper chromatography of each fraction of wattle and quebracho tannins obtained from the chromatographic separation on thick sheets showed that each fraction was broad in direction 1 (butanol–acetic acid–water or water-saturated butan-2-ol) but narrow in direction 2 (2% acetic acid). R_F values of the bands in direction 2 correspond approximately to those of the same bands when cut from the thick sheets.

The anthocyanidins generated from each fraction of black-wattle tannins contained robinetinidin, fisetinidin and an orange–yellow pigment. The proportions of these pigments varies with the different fractions. Quebracho tannins yield mainly fisetinidin from each fraction. The yields of anthocyanidins from the various fractions are given in Table 6. These are free of 'non-tannin' carbohydrates, and their values are therefore comparable.

DISCUSSION

Since the first recognition of the widespread distribution of leuco-anthocyanins by Robinson & Robinson (1933, 1934) and the use of qualitative colour reactions for the identification of anthocyanidins, the method of recognizing these classes of compounds was enhanced by the use of paper chromatography. Bate-Smith (1954) used this technique for identifying anthocyanidins generated from leuco-anthocyanins conforming to the delphinidin and cyanidin types, and Roux (1957*a*, *b*) extended the range of anthocyanidins, their

aluminium complexes (Geissman, Jorgenson & Harborne, 1953; Geissman & Jurd, 1955) and a new solvent system being used to assist in their differentiation.

Roux (1957*b*) also used a new chromogenic reagent, toluene-*p*-sulphonic acid, for locating leuco-anthocyanins on paper chromatograms and for differentiating between monomeric and polymeric leuco-anthocyanins. The reagent gives various scarlet colorations with different monomeric (Table 2) and naturally occurring polymeric leuco-anthocyanins, and from their relative positions on paper chromatograms the monomeric (R_F 0·47–0·55 in 2% acetic acid) and polymeric (R_F 0–0·45) forms may be differentiated. Monomeric leuco-anthocyanins, assuming adequate separation has been achieved, will also appear as discrete pink spots, whereas complex leuco-anthocyanins are usually represented as broad pink bands extending often continuously from R_F 0·45 to the origin. The use of these methods is demonstrated in the examination of the heartwoods of a large number of species, where the high proportion of monomeric forms present amongst certain genera of the family Leguminosae is of special interest (Table 3).

For quebracho extract, the overall development of pink colorations with toluene-*p*-sulphonic acid reagent in almost all those areas on two-way paper chromatograms where reducing tannins are located shows the wide distribution of leuco-anthocyanins throughout the tannin fraction. A close interrelationship between various tannin constituents with different mobilities on paper chromatograms also appears to exist, as independent of their rate of migration and their molecular weights (Table 5) each fraction contains leuco-fisetinidins (Table 6). The complex leuco-fisetinidins all have lower R_F values in 2% acetic acid than monomeric leuco-fisetinidins, and undoubtedly possess greater structural complexity (mol.wt. 790–2350) than the monomeric form (mol.wt. 290).

The lower yield of fisetinidin from quebracho leuco-anthocyanins (5·6%) compared with 7:3′:4′-trihydroxyflavan-3:4-diol (23·8%) indicates that approx. 24% of the 'fundamental units' present in the tannins possibly are of the 'leuco-fisetinidin type'. Considering the even distribution of leuco-anthocyanins throughout the tannins on two-way paper chromatograms, this figure may be on the low side. This evidence, however, demonstrates the C_6–C_3–C_6 nature of a fairly high proportion of the 'fundamental units' present in quebracho tannins.

The work on wattle extract closely parallels that on quebracho, but the leuco-anthocyanin mixture present in wattle is more complex. Whereas the leuco-anthocyanins of quebracho extract are mainly leuco-fisetinidins, black-wattle tannins contain leuco-robinetinidins predominantly, but also considerable proportions of leuco-fisetinidins and other constituents capable of forming an unidentified orange–yellow pigment. Furthermore, whereas the leuco-fisetinidins of quebracho extract are fairly evenly distributed throughout the reducing tannins on paper chromatograms, the mobile leuco-robinetinidins, leuco-fisetinidins and other colour-producing compounds of wattle extract may be differentiated amongst the mobile tannins, although a considerable amount of overlap of constituents occurs. Such overlap is to be anticipated in the migration of highly polar units of large molecular weight and high affinity for cellulose, on paper chromatograms. The proportion of fisetinidin generated from the extracts of barks from individual trees reputed to be authentic examples of *Acacia mollissima* varies somewhat. Bark extracts of the closely related species, silver wattle (*A. dealbata*) and green wattle (*A. decurrens*), furnish the same anthocyanidins but in both instances the percentage of fisetinidin generated appears to be higher compared with black wattle. The proportion of leuco-anthocyanins present in the complex wattle tannins (molecular weight 550–3250) appears to be of the same order as those present in quebracho (Table 4).

Both wattle and quebracho tannins on alkaline fusion yield mainly those phenolic nuclei (resorcinol and protocatechuic acid from quebracho tannins, and resorcinol, gallic and protocatechuic acids from wattle; Freudenberg & Maitland, 1934; Roux, 1952) which are potentially present in the anthocyanidins generated from the extract.

For studying the range and distribution of molecular weights in relation to leuco-anthocyanin distribution in wattle and quebracho tannins, the method of fractionating the tannins on cellulose sheets was used. This method has the disadvantage that recoveries of the polyphenolic tannins by elution is not quantitative. Nevertheless, by using a paper-chromatographic method, the fractions isolated may be linked directly to the distribution of complex leuco-anthocyanins on two-way paper chromatograms.

Wattle tannins range in molecular weight mainly from 550 to 1630, but also contain a small fraction of much higher (3240) average molecular weight. Quebracho tannins are evenly distributed throughout the range 800–2350, but the molecular weight of quebracho tannins in each R_F fraction is comparatively higher than the value for the corresponding wattle fraction (Table 5).

The complex wattle and quebracho tannins both show a stepwise increase of molecular weight with increase of affinity for the cellulose substrate in aqueous medium. As wattle tannins show parallel affinity characteristics for both cellulose and

collagen during chromatographic separations (Roux, 1955), it follows that with increase of molecular weight tannins show greater affinity also for collagen. The relationship between affinity and molecular weight is important in the interpretation of the chromatographic behaviour of 'condensed' tannins, and is also of interest in studying the behaviour of tannins towards collagen, where the affinity effects are believed to be mainly due to hydrogen bonding (Shuttleworth & Cunningham, 1948; Ellis & Pankhurst, 1954).

From the two-dimensional chromatography of each fraction and also from the regular increase of molecular weight with decrease of R_F value may be gauged the efficiency of the fractionation procedure. The relatively even distribution of leuco-anthocyanins in these fractions throughout the whole R_F range (Table 6) confirms similar evidence obtained by spraying two-dimensional chromatograms of the tannins with the toluene-*p*-sulphonic acid reagent, and shows that complex leuco-anthocyanins of varying molecular weight, molecular complexity and affinity for various substrates are present in both wattle and quebracho tannins.

SUMMARY

1. Examination of the heartwood extractives of 144 'hardwood' species by methods developed by Roux (1957*a*, *b*) show that 33% give strongly positive reactions for leuco-anthocyanins. Lower R_F polymeric forms predominate, and in only 12%, mainly amongst the Leguminosae, is evidence found of the presence of monomeric leuco-anthocyanins.

2. Black-wattle and quebracho tannins have been shown to be relatively rich in complex leuco-robinetinidins and leuco-fisetinidins (Roux, 1957*b*). Comparison of the yields of anthocyanidins from complex naturally occurring leuco-anthocyanins and from synthetic monomeric leuco-anthocyanins indicates that about 25% of the 'fundamental units' present in these complex tannins are of the leuco-anthocyanin type.

3. For each tannin mixture, a gradational increase of molecular weight with decrease of R_F value of the tannins was found, when separated on paper chromatograms in 2% acetic acid. The molecular weights of wattle tannins fall mainly in the range 550–1630, although a small fraction of much higher (3240) molecular weight is also present. Values for quebracho tannins fall in the range 800–2350.

4. Similar proportions of leuco-anthocyanins are present throughout the whole molecular-weight range of both tannins, indicating the presence of leuco-anthocyanins (e.g. leuco-robinetinidins and leuco-fisetinidins) of increasing complexity from the higher R_F (0·5–0·6) to the lower R_F (0) regions.

5. With increase in molecular weight condensed tannins show a parallel increase in 'affinity' for cellulose, and also for collagen (Roux, 1955).

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The Reconstitution of Elastin from a Soluble Protein derived from *ligamentum nuchae*

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The object of this investigation has been to study the conversion of soluble elastin from ox *ligamentum nuchae* into a form resembling native elastin. Study of the precipitation of collagen fibrils from collagen solutions has provided an insight into the formation of collagen *in vivo*, but the possibility of reconstituting elastin from elastin solutions has been neglected.

Purified elastin from ox *ligamentum nuchae* dissolves slowly in 0·25 M-oxalic acid at 100° to give a water-soluble polydisperse product whose number-average molecular weight is about 60 000 (Adair, Davis & Partridge, 1951; Partridge, Davis & Adair, 1955). This can be divided into an α-fraction (mol.wt. 60 000–84 000) which forms a precipitate on heating, and a β-fraction (mol.wt. 5500) which does not. The precipitate is composed of liquid droplets, rich in protein (i.e. a coacervate), which disappear when the solution is cooled again. Reversible coacervation is also a property of solutions of elastin solubilized by boiling with 40% (w/v) urea solution (Bowen, 1953). The lowest temperature at which coacervation of elastin solubilized with oxalic acid can be observed depends on pH and ionic strength, showing a minimum near the isoelectric point (pH 4·8 at ionic strength 0·01, pH 3·9 at ionic strength 0·1; Partridge *et al.* 1955).

The present investigation shows that the reversal of coacervation on cooling may be arrested under suitable conditions and the coacervate converted into a gel whose properties are similar to those of the original elastin.

MATERIALS AND METHODS

Preparation of soluble elastin. Fresh ox *ligamentum nuchae* was freed from adhering tissue, cut into 5 mm. cubes and minced in a power mincer, solid CO_2 being used to chill the material (Hall, 1955). Preparation of purified elastin from the minced ligament followed the procedure of Partridge *et al.* (1955) except that, instead of using a stainless-steel hammer mill to powder the material, it was gently rubbed through a series of standard sieves. At the end of the preparation 80% of the elastin passed through a sieve (150 mesh/in.) and this fraction was used. It appeared under the microscope as a mixture of short fibres and small bundles of fibres, and the remaining 20% consisted of larger bundles of fibres.

Samples of purified elastin were dissolved by the following methods: (i) Heating with 0·25 M-oxalic acid as described by Partridge *et al.* (1955) in their preparation of the 'primary extraction product'. Soluble elastin prepared by this method is referred to below as 0_0.

(ii) Serial extraction with 0·25 M-oxalic acid at 100° as described by Partridge *et al.* (1955). Six fractions were obtained and the proteins isolated from them are referred to below as 0_1–0_6. 0_1 consists mainly of β-elastin and successive fractions of this series consist of mixtures of β-elastin and α-elastin with gradually increasing proportion of α-elastin (Partridge *et al.* 1955).

In each method the resulting solutions were dialysed against distilled water and freeze-dried. The carbohydrate contents (as glucose) of the products 0_1–0_6 determined by the anthrone method of Seifter, Dayton, Norrie & Muntwyler (1950) were 0·8, 0·2, 0·1, 0·0, 0·1 and 0·1% respectively.

Special chemicals. The sample of elastase was the proteolytic elastase fraction E_2 prepared by Dr D. A. Hall (Hall, 1957*a*). Crystalline trypsin was supplied by L. Light and Co. Ltd.

Methods. The temperature of coacervation was determined as described by Partridge *et al.* (1955).

Protein concentrations were determined either by the micro-Kjeldahl nitrogen estimation, a nitrogen content of 16·3% being assumed, or, for comparative purposes, by determining the u.v.-light absorption at a wavelength of 275 mμ of solutions in 0·1 N-NaOH with a Unicam SP. 500 spectrophotometer.

Load–extension curves were determined on strips of tissue or gel 1–2 cm. long and about 0·02 cm.^{2} in cross-sectional area. A strip was mounted between glass hooks and suspended in a glass tube (length 15 cm., diam. 1 cm.) containing water whose temperature was kept constant by circulating through a jacket water from a thermostat. When tissue was studied the upper end of the strip was fixed. Load was applied by adding weights to a pan connected to the lower end of the strip by a length of fine stainless-steel wire which passed through a glass capillary in the bottom of the tube. With strips of gel the loads involved were much lower than with tissue and the procedure was therefore modified. The lower end of the strip was kept fixed, relative to the tube, and the upper end attached to the beam of a dial-reading torsion balance (0–500 mg.). Load was applied by rotating the balance arm. The balance beam and upper end of the strip were then restored to their zero position by lowering the tube. In both methods the length of the strip was measured with a cathetometer.

All solutions were freshly prepared before use and the work was done under normal conditions of laboratory cleanliness. In view, however, of the long periods of

incubation in some of the experiments these were repeated with toluene as preservative. The results did not differ significantly from those obtained without toluene.

EXPERIMENTAL AND RESULTS

Precipitation of elastin by heat

Solutions of 0_0 and 0_3–0_6 (1%, w/v, in acetate buffer, pH 5·0, ionic strength 0·2) heated to 50° for 5 min. showed coacervation, which disappeared on cooling. When heating was continued for 16 hr. before cooling, the coacervate, which had then collected at the bottom of the vessel as a viscous layer, did not redissolve completely but when stirred coagulated into an elastic mass. The amount of elastin which precipitated and the solubility of the precipitate in buffer depended on temperature and time of heating, pH and ionic strength of the solution and type of soluble elastin, as shown below.

Temperature. A solution of 0_0 (1%, w/v, in acetate buffer, pH 5·0, ionic strength 0·15) was kept at 30° for 16 hr. After cooling, the supernatant was poured off and kept at 35° for 16 hr. The procedure was repeated at higher temperatures. The precipitates obtained at the temperatures above 30° were dissolved in buffer, dialysed, freeze-dried and weighed. The precipitate at 30° did not dissolve in the buffer but did dissolve in 7 M-urea. This solution, too, was dialysed, freeze-dried and weighed. The results (Table 1) show that most precipitation occurred in the range 30–35°, but only the small amount which precipitated in the lowest temperature range was insoluble in buffer.

Table 1. *Extent of precipitation of elastin at different temperatures*

Elastin 0_0, 1% (w/v) in acetate buffer, pH 5·0; ionic strength 0·15.

Temperature range	Elastin precipitated (% original wt.)
18–30°	11
30–35	15
35–40	9
40–50	14
50–60	4

Time of heating, pH and type of elastin. Samples (2 ml.) of solutions (1%, w/v) of 0_3, 0_4, 0_5 and 0_6 in buffers of pH 3·2, 5·1 and 7·0 and ionic strength 0·2 were heated to 40° in stoppered tubes for different times, cooled to room temperature (18°) for 1 hr., stirred briefly to disperse the precipitate, centrifuged for 5 min. and 1 ml. of the supernatant was diluted to 10 ml. with 0·1 N-NaOH. The extent of precipitation was calculated from the u.v.-light absorption of these dilutions and of similar dilutions of the original solutions. The results (Table 2) show, as was to be expected from the work of Partridge *et al.* (1955), that only a small amount of elastin precipitated at pH 7·0. Larger amounts precipitated at lower pH but there was no significant difference between the amounts formed at pH 3·2 and 5·1. In each case precipitation was near its maximum extent at 5 hr. and little further change resulted from heating for 16 hr. The data indicate that for the same pH and time of heating the extent of precipitation increased as the fraction number increased. This is consistent with Partridge's observation that as the fraction number increases the proportion of the coacervate-forming α-fraction of elastin increases. All further experiments on heat precipitation were therefore done on the fraction 0_6, which is rich in α-elastin.

Ionic strength and protein concentration. The extent of precipitation of 0_6 from solution in acetate buffer (pH 5·0) at 40° was the same at ionic strengths 0·15 and 0·07 and at protein concentrations 1·0 and 0·5%.

Solubility of precipitates. The precipitates dissolved slowly if kept in buffer at 4° for a long time. Precipitates were prepared by heating samples (3 ml.) of 1% (w/v) solutions of 0_6 in acetate buffer, pH 5·0, at 40° for various times. The extent of precipitation was determined and the precipitates were left in contact with 3 ml. of fresh buffer for 4 days at 4° with occasional stirring. The amount of precipitate which had redissolved was measured. The results (Table 3) indicate that precipitates prepared by heating for 23 hr. dissolved much more slowly than those prepared by heating for shorter periods, and also that elastin precipitated at ionic strength 0·075 was more soluble than that precipitated at ionic strength 0·15.

Table 2. *Extent of precipitation of elastin in different buffers for different times of heating*

Buffers: pH 3·2, glycine hydrochloride; pH 5·1, sodium acetate; pH 7·0, sodium phosphate. Ionic strength 0·2. Temperature 40°.

Elastin fraction		0_3			0_4			0_5			0_6		
Time of heating (hr.)		1	5	16	1	5	16	1	5	16	1	5	16
Elastin pptd. (% original wt.)	pH 3·2	2	13	14	23	—	32	27	47	31	18	38	36
	pH 5·1	4	16	15	9	25	25	19	49	50	33	41	32
	pH 7·0	5	9	3	2	10	6	6	12	6	5	1	0

Table 3. *Solubility of precipitated elastin*

Elastin 0_6, 1% (w/v) in acetate buffer, pH 5·0. Temperature 40°.

Ionic strength	Time of heating (hr.)	Elastin precipitated (% of original amount in solution)	Elastin redissolved in fresh buffer after 4 days at 4° (% of the precipitated elastin)	Elastin redissolved in fresh buffer after 4 days at 4° (% of original amount in solution)
0·15	3	20	86	17
0·15	23	49	8	4
0·075	6	39	60	23
0·075	23	51	29	15

Precipitates prepared by heating solutions of 0_6 at pH 5·0 for 16 hr. and ionic strength 0·15 were soluble in formic acid (98–100%), in dichloroacetic acid and in *m*-cresol. If, however, they were washed with water and then with ethanol and the ethanol was removed *in vacuo*, they became insoluble in these media although they swelled markedly.

Precipitation by other methods

The soluble elastins dissolved readily in formic acid (98–100%), and more slowly in dichloroacetic acid, to give viscous solutions. When the solutions in formic acid were diluted with 3 vol. of water at room temperature coacervates formed which settled to the bottom of the flask to form viscous layers after 16 hr. They coagulated on stirring to give an elastic mass. When solutions in dichloroacetic acid were diluted in the same way a fibrous friable precipitate formed, which did not coagulate on stirring.

Gel-like precipitates could also be prepared by acidifying a solution of 0_6 in 0·2 M-NaCl with N-HCl to pH 1·0 at room temperature and allowing to stand for 16 hr. The amount of elastin precipitated by this procedure was increased by the presence of up to 20% of ethanol, but when there was more than 60% of ethanol present no precipitate formed.

The precipitates formed by these methods were insoluble in water even after prolonged washing but dissolved readily in formic acid. In view, however, of difficulty experienced in removing acid completely from precipitates prepared by these methods further study was restricted to heat-precipitated elastin.

Some properties of heat-precipitated elastin

Samples of heat-precipitated 0_6 were prepared for physical measurements as follows: A solution (80 ml.) of 0_6 (0·6%, w/v, in acetate buffer, pH 5·0, ionic strength 0·15) was heated to 55° in a stainless-steel tube, internal diam. 3 cm., whose lower end was closed by a removable polystyrene disk. After 16 hr. most of the coacervate had collected on the disk and after cooling was removed from it with a scalpel as a yellow translucent gel. The gel was carefully washed with water and then with aqueous ethanol, the ethanol concentration gradually being increased to 100%. The gel was finally dried *in vacuo* to give an opaque, brittle disk about 1 mm. thick. When immersed in ethanol or water it swelled and became elastic.

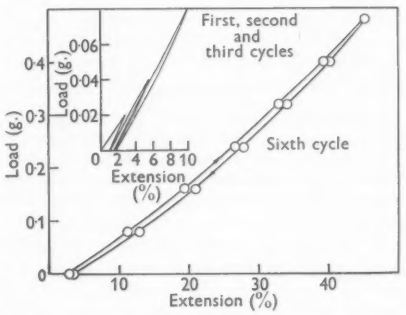

Fig. 1. First, second, third and sixth cycles of a series of load–extension cycles for a strip of heat-precipitated elastin of cross-sectional area about 0·02 cm.² immersed in water at 18°. Each increment of load and measurement of length took about 1 min. Thus the time taken for the sixth cycle was approx. 12 min.

Elastic properties. A disk of precipitate was soaked in ethanol, cut into strips and washed thoroughly with water, and load–extension curves were determined. Fig. 1 shows a series of load–extension cycles in which the maximum extension was gradually increased. There was slight hysteresis, but even after 50% extension the strip returned almost to its original length. The Young's modulus of the strip was about 5×10^4 dynes/cm.² and its extension at break was about 120%. The thermo-elasticity of precipitated elastin was studied by determining load–extension curves of a single strip at different temperatures. The variation of tension with temperature at constant length calculated from these data is shown in Fig. 2. The strip had previously been stretched 30% and allowed to retract in order to eliminate the small irreversible extension shown in Fig. 1.

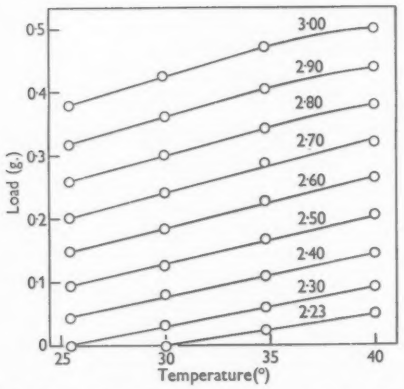

Fig. 2. Load–temperature curves at constant length derived by interpolation from load–length curves for a single strip of heat-precipitated elastin immersed in water at four different temperatures. Approximate cross-sectional area 0·02 cm.^{2} The length in cm. is shown against each curve.

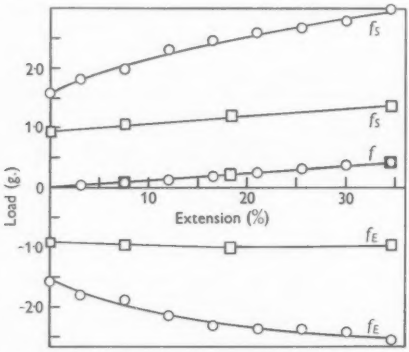

Fig. 3. Analysis of load (f)–extension curves of elastin into contributions (f_E and f_S) due to changes of internal energy and entropy. Data are shown for heat-precipitated elastin at 30° (○), calculated from data given in Fig. 2, and for collagen-free elastin at 30° (□), calculated from similar unpublished data. In order to facilitate comparison, the values of f, f_E and f_S for collagen-free elastin have been multiplied by $5{\cdot}5 \times 10^{-3}$ so that its load–extension curve is coincident with that of heat-precipitated elastin.

Thermodynamic analysis of elasticity. When any material is stretched the equilibrium tension (f) may be expressed in the following form (see, for example, Treloar, 1949):

$$f = (\partial E/\partial L)_{T,\,V} - T(\partial S/\partial L)_{T,\,V} = f_E + f_S,$$

where f_E and f_S are the contributions to f due respectively to change of internal energy (E) and entropy (S) with length (L) at constant temperature (T) and volume (V). Since

$$(\partial S/\partial L)_{T,\,V} = -(\partial f/\partial T)_{L,\,V}$$

knowledge of f, T and $(\partial f/\partial T)_{L,\,V}$ enables one to calculate f_S and f_E. Application of these equations to the data shown in Fig. 2 leads to the values of f_S and f_E shown in Fig. 3. The figure includes data derived in a similar way from experiments on a strip of ox *ligamentum nuchae* from which the collagen had been removed by treatment with boiling 1 % (v/v) acetic acid.

X-ray diffraction. Dr W. G. Horton of Bradford Technical College examined the dried gel by wide-angle X-ray diffraction with the beam perpendicular to the plane of the gel. A pattern characteristic of an amorphous structure with diffuse rings at 4·4 and 9 Å was obtained.

Solubility. Like the other dried elastin precipitates the dried elastin gel was insoluble in aqueous buffer solutions, formic acid (98–100 %), dichloroacetic acid and *m*-cresol at room temperature. To test the solubility of the dried elastin gel in hot water or hot 1 % (w/v) acetic acid, in which solvents native elastin is insoluble, samples (100 mg.) were boiled under reflux with 5 ml. of solvent. At 6 hr. intervals undissolved material was removed by centrifuging and treated with a further 5 ml. of solvent, and the protein content of the extract determined. After six extractions water had dissolved only 5 % of the material. Acetic acid dissolved 50 % in the first extraction and after six extractions over 90 % had dissolved. Similar experiments with purified elastin showed that six extractions with either solvent dissolved less than 5 % of the material.

Susceptibility to enzymes. Samples of precipitated elastin (50 mg.) were immersed in 3 ml. of (i) a solution of the proteolytic elastase fraction E_1 (Hall, 1957*a*) (0·03 mg./ml. in borate buffer, pH 8·4), or (ii) a solution of trypsin (0·03 mg./ml. in phosphate buffer, pH 7·1). Elastase dissolved the material completely in 1 hr. at room temperature (18°), whereas a similar weight of purified elastin dissolved only after several hours at 37°. Trypsin dissolved precipitated elastin completely within 16 hr. at 37° but under the same conditions dissolved only 4 % of a sample of purified elastin. Neither buffer on its own dissolved precipitated elastin.

Staining properties. Precipitated elastin appeared globular under the microscope. When stained with the Weigert stain some areas stained red like the whole of the elastin fibres of native *ligamentum nuchae*, but there was a greater proportion of dark-staining areas. Dark-staining areas were also observed in purified elastin but to a smaller extent.

DISCUSSION

Heat-precipitated elastin resembles purified elastin in the following properties: (i) both show long-range elasticity, the same shape of load–extension curve and approximately the same extension at break (Astbury, 1940; Burton, 1954; Wood, 1954); (ii) analysis of their thermo-elastic behaviour shows that the elasticity of both materials is of a type found in only a few fibrous proteins (Meyer & Ferri, 1936; Wöhlisch, Weitnauer, Grüning & Rohrback, 1943; Lloyd & Garrod, 1946). The tension is composed of a high, positive, entropy contribution and a high, negative, internal-energy contribution; (iii) both materials give similar X-ray-diffraction patterns (Astbury, 1940); (iv) both are insoluble in all the solvents considered except hot 1% (w/v) acetic acid, which dissolves precipitated elastin; (v) both are dissolved by elastase (Balo & Banga, 1950; Hall, 1957*a*) but precipitated elastin is dissolved more rapidly; (vi) both stain in a similar manner with the Weigert stain.

The two materials differ in the following respects: (i) the Young's modulus of precipitated elastin is only about one-hundredth of that of purified elastin (Burton, 1954); (ii) precipitated elastin is dissolved by hot 1% (w/v) acetic acid and by trypsin but purified elastin is not (Balo & Banga, 1950; Partridge & Davis, 1955); (iii) precipitated elastin is non-fibrous, unlike purified elastin.

The similarity between the X-ray diagram, insolubility and elasticity of precipitated and purified elastin, together with the fact that α-elastin has an amino acid analysis almost identical with that of purified elastin (Partridge & Davis, 1955), strongly suggests that in the two materials the molecules have essentially the same configuration. To this extent heat-precipitated elastin may be termed reconstituted elastin. The X-ray pattern indicates an amorphous structure and distortion of such a structure by stretching would be expected to give rise to the high positive-entropy tension that is in fact observed. The high negative internal-energy tension might be due to molecular repulsions between the bulky non-polar amino acid side chains, of which there are many in elastin (Partridge & Davis, 1955). In this respect elastin resembles casein (Waugh, 1954), fibres of which also show hyperentropic elasticity (Woods, 1946). Recently Ramachandran & Santhanam (1957) have suggested that elastin has a small degree of ordered structure, and Flory (1956) has shown that hyperentropic elasticity might arise from such a structure.

The differences between reconstituted and purified elastin indicate that although the arrangement of the molecules appears to be the same in the two materials, some factor or factors which stabilize purified elastin are lacking in reconstituted elastin. The solubilization of elastin by oxalic acid is accompanied by the breaking of peptide linkages and release of aspartic and possibly glutamic acid (Adair *et al.* 1951). End-group studies (Partridge & Davis, 1955) indicate that the number-average chain length of elastin may be reduced 100-fold during solubilization. The length of the polypeptide chains in reconstituted elastin is therefore probably much lower than in purified elastin and this would be expected to result in differences of Young's modulus, solubility in the solvents studied and resistance to the solubilizing action of proteolytic enzymes such as are observed. Breaking of cross-linkages of purified elastin (Partridge & Davis, 1955) during solubilization would be expected to have a similar effect.

Those properties held in common by reconstituted and purified elastin must be characteristic of the protein of which both are composed. The results indicate therefore that in purified elastin these properties are not due to the fibrous nature of the material. In view of the way in which it is precipitated, reconstituted elastin probably does not have a fibrillar fine structure like that observed in native elastin (Lansing, Rosenthal, Alex & Dempsey, 1952; Hall, Reed & Tunbridge, 1954). Nor is it likely to consist of a protein core surrounded by a sheath of different composition, as has been suggested for native elastin (Hall, Reed & Tunbridge, 1952; Hall, 1957*b*). The common properties (elastic behaviour, insolubility, staining with the Weigert stain, X-ray-diffraction pattern) cannot therefore be due primarily to either of these two-component structures. Such structures might, however, contribute to the stability of native elastin and their importance for other properties is not excluded (see, for example, Saxl, 1957).

Consideration of the conditions of heat precipitation shows that the longer the time of heating the greater is the extent of precipitation and the lower the rate at which the precipitated elastin redissolves. This indicates that heat coacervation, which is rapid and reversible, is followed by a slower process which retards resolution. Partridge *et al.* (1955) suggested that coacervation of elastin is due to unfolding of the molecules and their subsequent aggregation. The slow process is probably increase in the extent of aggregation. Covalent linkages are probably not formed to any great extent during this process since the resulting precipitates dissolve in hydrogen-bond-breaking agents such as formic acid and dichloroacetic acid and become insoluble in these solvents only after being washed with ethanol.

The present work shows that a soluble degradation product of elastin can be converted into an insoluble form, having many of the characteristic properties of the original elastin. The way in which elastin is formed *in vivo* is not known with certainty but it is at least a possibility that it is formed from a soluble precursor by a mechanism similar to reconstitution.

SUMMARY

1. The high-molecular-weight α-fraction of soluble elastin prepared from ox *ligamentum nuchae* (Adair *et al.* 1951) becomes insoluble on prolonged heating of aqueous solutions under certain conditions.

2. Heat-precipitated elastin has many of the physical properties of purified elastin and it is concluded that the protein molecules have essentially the same configuration in the two materials.

3. Differences between heat-precipitated (reconstituted) elastin and purified elastin may be attributed to degradation of the latter when it is converted into soluble elastin.

4. The precipitation of elastin appears to consist of two processes, namely a rapid one which may involve configurational changes and results in coacervation and a slow one which is probably due to aggregation of the protein molecules.

5. The bearing of the results on the structure of elastin and its fibrogenesis is discussed.

I wish to thank Miss H. Saxl for studying the histology of purified and precipitated elastin and Dr W. G. Horton for examining precipitated elastin by X-ray diffraction. I also wish to thank Miss H. Saxl, Dr D. A. Hall and Professor R. E. Tunbridge for helpful discussion and criticism and Miss J. Waddington for technical assistance.

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The Site of Protein Synthesis in *Bacillus megaterium*

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In recent years much work has been done on the incorporation of isotopically labelled amino acids into the various fractions of mammalian cells. It has been found that the ribonucleoprotein particles associated with the microsomal fraction become labelled at a very early stage (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Keller, Zamecnik & Loftfield, 1954; Littlefield, Keller, Gross & Zamecnik, 1955; Simkin & Work, 1957; Cohn & Butler, 1957), although evidence for a preliminary activation process in the 'soluble fraction' has been given (Hoagland, Keller & Zamecnik, 1956; Hoagland, Zamecnik & Stephenson, 1957). On the other hand, little information has been obtained about the sites of protein synthesis in the bacterial cell. It has, however, been shown that in *Torulopsis utilis* absorbed amino acids do not exist in the free state at all but are bound in such a way

that they are not freely exchangeable with amino acids in the surrounding medium (Cowie & Walton, 1956), and Gale & Folkes (1955) found that a cell-envelope fraction from *Staphylococcus aureus* was capable of developing enzyme activities and of incorporating labelled amino acids into protein. These two reports indicated that the cell wall or cytoplasmic membrane might play an important part in the synthesis of protein by micro-organisms.

Bacillus megaterium seemed to us a suitable micro-organism for studies of the site of protein synthesis in view of the work of Weibull (1953*a*, 1955). He had shown that it is easily possible to remove the cell wall by treatment with lysozyme, leaving, when suitable precautions are taken, the protoplasm of the cell in the form of spherical protoplasts.

It has been shown (McQuillen, 1956) that these protoplasts are able to synthesize proteins, nucleic acids and enzymes, including adaptive enzymes; they can also grow and divide and allow the development of endospores. When shaken violently, or placed in media of low osmotic pressure, they can be lysed, with the resultant liberation into the medium of the soluble cell constituents. Two structures then remain: empty vesicles or 'ghosts' derived from the cytoplasmic membrane that originally surrounded the protoplasts and small granules. The membranes and granules may be isolated by differential centrifuging (Weibull, 1953*b*), and the separated granules appear to consist largely of polymerized β-hydroxybutyric acid. If the membrane–granule fraction is sedimented under the conditions described in this paper, the clear supernatant fraction (cytoplasm) deposits little further sediment when centrifuged for 30 min. at 100 000 *g*, so that there appears to be no close equivalent of the mammalian microsome fraction present (Hunter, Crathorn & Butler, 1957).

In the experiments reported here a study has been made of the incorporation of labelled amino acids into the cell wall, the cytoplasmic membrane and the supernatant fractions. The incorporation has been studied in (1) the intact bacterium; (2) intact and broken protoplasts; (3) the membrane and supernatant fractions of the protoplasts. A preliminary account of part of this work has been given (Crathorn & Hunter, 1958).

METHODS

Organism. We are indebted to Dr K. McQuillen for supplying the original strain KM of *Bacillus megaterium*. It has been maintained by frequent subculture on peptone–agar slopes. By progressive selection, a strain has been isolated that forms very stable protoplasts when treated with lysozyme. These can be treated almost as whole cells when suspended in neutral media containing 0·5 M-phosphate and can then be disintegrated only after very violent shaking.

The organism was usually grown at 30° in a glucose–salts solution (McQuillen, 1955) medium, although on some occasions the glucose was replaced by 0·5 % (w/v) peptone (British Drug Houses Ltd., Poole, England) without apparently affecting any of the biochemical properties studied here. Large batches for experimental purposes were prepared in aerated Roux bottles or 5 l. shake flasks.

Experiments with whole cells. Whole cells from large batches grown overnight were harvested and washed, suspension densities being determined from the absorption at 420 mμ on a Unicam spectrophotometer (model SP. 600) previously calibrated against the organism. For tracer experiments the cells were resuspended in the glucose–salts medium and re-incubated aerobically at 30° for 1 hr. before adding any [^{14}C]amino acid. After further aerobic incubation for the required length of time, the cells were sedimented and resuspended in a medium containing 0·5 M-sodium citrate, 0·5 % (w/v) of casein digest (Bacto-Casamino acids; Difco Laboratories Inc., Detroit, Mich., U.S.A.), and 0·02 % of lysozyme. Protoplasts were collected by centrifuging after standing overnight at 2°, and the digested cell-wall material was obtained in the supernatant medium. The protoplasts were finally lysed by pouring into 4 vol. of water and the fraction containing the cytoplasmic membrane and granules was collected by sedimentation at 10 000 *g* for 10 min. In some experiments it was found necessary to sediment at 20 000 *g* for 20 min. to obtain a firm pellet.

The supernatant fraction from this last centrifuging contained the cytoplasm of the cell diluted with the suspension medium. It was found that estimations of the radioactivity of protein in this fraction were in many cases little affected if the cell-wall digest and free amino acids were also present, and in some experiments a better control of timing was attained by pouring the original cell suspension directly on to ice at the end of the period of incubation with the labelled amino acid. Lysozyme was added to a final concentration of 0·02 % and the cytoplasmic membranes were isolated as before after standing overnight at 2°.

In some cases the cytoplasmic-membrane fractions were further treated with ethylenediaminetetra-acetic acid (EDTA) (1 % sodium salt; pH 7) at 30° for 1 hr. This had the effect of solubilizing many of the granules and bringing into solution about one-third of the protein and some of the deoxyribonucleic acid (DNA). The actual amount of DNA solubilized in this way was variable (35–65 %). Vennes & Gerhardt (1956) claim that it is possible to remove DNA completely from the cytoplasmic membranes of *B. megaterium* by treating protoplasts with a mixture of EDTA and deoxyribonuclease, but under conditions where the activity of the enzyme was probably very small.

Experiments with protoplasts. Protoplasts were prepared by gently shaking suspensions of whole cells at a density of 1 mg./ml. (dry wt.) in air at 30° in a medium containing 0·5 M-sodium citrate, 0·5 % of casein digest and 0·02 % of lysozyme. The complete conversion into protoplasts usually took about half an hour under these conditions, sometimes much longer. On occasion, suspensions were obtained that were partially or completely insensitive to the action of lysozyme under these conditions. We can offer no

explanation of this phenomenon; fortunately it is not of very frequent occurrence. The protoplasts were sedimented and resuspended for incorporation experiments in 'C' solution (McQuillen, 1955) containing, in addition, 0·5 M-KH_2PO_4 and 1% of glucose, the whole brought to pH 7 with sodium hydroxide. In this medium they were very stable and could be shaken vigorously enough to maintain adequate aeration without appreciable lysis taking place. It is always advisable, however, to follow closely by microscopic examination, any metabolic experiments with whole protoplasts.

After shaking at 30° in this medium for 1 hr. labelled amino acids were added and any further period of incubation of the protoplasts could be defined clearly by pouring the whole or part of the suspension directly on to 4 vol. of ice–water, when instantaneous lysis occurred. The cytoplasmic membrane fraction was then separated at once from the cytoplasmic fraction by centrifuging at 10 000–20 000 *g* for 10–20 min.

Experiments with broken protoplasts and isolated membranes. Thick suspensions of protoplasts (20 mg./ml.) in phosphate–'C'–glucose medium, detailed in the previous section, were broken by rapid oscillation in a Mickle tissue disintegrator. The suspension was then diluted 20-fold with the same medium and incubated aerobically at 30° for 30 min. ^{14}C-Labelled amino acid was then added in order to study the incorporation of the amino acid into protein and the incubation continued for varying times, eventually being stopped by rapid cooling. The membrane fraction was then separated from the cytoplasm and incubation medium by sedimentation at 20 000 *g* for 20 min.

In other experiments, the cytoplasmic membranes were separated from the cytoplasm by centrifuging and the incorporation of [^{14}C]amino acid was studied in the two separated fractions. The protoplasts could also be broken by diluting twice with water and shaking vigorously by hand for 2 min., and the properties of the isolated membranes and cytoplasm thus obtained were very similar to those of the corresponding fractions obtained by the previous treatment. For incorporation studies on the isolated membranes, they were usually resuspended in the inorganic 'C' solution with 1% of glucose added.

When transfer experiments were carried out with cytoplasmic membranes labelled *in vitro*, the labelling medium was removed and the washed membranes were then reincubated aerobically with non-labelled cytoplasm at 30° for the appropriate period of time. The cytoplasm and the membranes were then separated once more by centrifuging at 20 000 *g* for 20 min.

Attempts to obtain incorporation of [^{14}C]amino acids into the protein of isolated cytoplasm were made by incubating the cytoplasm aerobically at 30° in the presence of various additives.

Estimation of nucleic acids. Ribonucleic acid (RNA) was determined in whole cells and subcellular fractions by the orcinol method (Cerriotti, 1955). DNA was determined by the indole method (Cerriotti, 1952). Samples of DNA and RNA which had been prepared from whole cells by a detergent method and separated by differential centrifuging (Hunter & Butler, 1956) were used as standard. These separated nucleic acids also served as standards in estimating the proportions of RNA and DNA in mixed nucleic acids extracted by treatment of various fractions with sodium dodecyl sulphate. Base analyses of extracted nucleic acids (Wyatt, 1951) were carried out after hydrolysis with perchloric acid (Marshak & Vogel, 1951).

Preparation of samples for radioactivity assay. Protein samples for counting were obtained from suspensions or solutions by adding aqueous trichloroacetic acid to a final concentration of 5% (w/v), and then removing lipids and nucleic acids from the precipitated proteins as described previously (Crathorn & Hunter, 1957). Where the total radioactivity of a given fraction was required it was evaporated to dryness at 90° and counted directly.

Assays of radioactivity. These were carried out where possible on 'infinitely thick' samples of protein or dried bacterial fractions plated on 1 cm.^{2} polythene disks (Popják, 1950), an EHM2S end-window Geiger–Müller counter in conjunction with an N529 scaling unit (manufactured by Ekco Electronics Ltd., Southend-on-Sea, Essex) being used. The counter was housed in a lead castle manufactured by E.R.D. Ltd., Slough, Bucks. Under these conditions a ^{14}C-labelled protein sample of specific activity 1 μc/g. would give a counting rate of about 500 counts/min. when plated at 30 mg./cm.^{2} (i.e. an efficiency of 1·2%). Samples of less than 'infinite' thickness were plated uniformly in the disks, and the counts obtained corrected to 'infinite' thickness by making use of an experimentally determined curve obtained with bacterial protein of known specific activity.

Materials. Generally labelled L-amino acids were supplied by the Radiochemical Centre, Amersham, Bucks. [1-^{14}C]Glycine (10·3 μc/mg.) and DL-[3-^{14}C]phenylalanine (12·0 μc/mg.) were synthesized in these Laboratories by Dr P. Brookes and Dr V. C. E. Burnop. All the [^{14}C]amino acids were used throughout this work without any dilution with unlabelled amino acid.

RESULTS

Experiments with whole cells. In order to determine the extent to which the cell wall participated in incorporation of amino acid, various ^{14}C-labelled amino acids were added to whole cells of *B. megaterium* growing in the full glucose–salts medium and incubations were continued for 5 or 30 min. before removing the cell walls with lysozyme. The results are summarized in Table 1; it can be seen that the specific activity of the cell-wall 'protein' is much lower than that of the protoplast protein in all cases. Of course, this might not have been true if any of the three amino acids most abundant in the cell wall of *B. megaterium* (alanine, glutamic acid and diaminopimelic acid) had been used. But it showed that the cell wall played no major part in the early stages of the synthesis of the bulk of the protein of the cell.

The rate of incorporation of ^{14}C-labelled amino acids into the proteins of subcellular fractions of the protoplasts was next studied. A typical result, in which [1-^{14}C]glycine was used, is shown in Fig. 1. For periods of incubation up to about 6 min. the protein of the membrane fraction has the highest specific radioactivity, but thereafter the cytoplasmic proteins become more highly radioactive.

Table 1. *Comparison of the incorporation of* ^{14}C*-amino acids into the proteins of cell walls and protoplasts*

Labelled amino acids (2 μc) were added to cells (100 mg. dry wt. at 1 mg./ml.) growing in a glucose–salts medium, and the incubation was continued as before for 5 or 30 min. The cells were then sedimented, washed and the cell walls digested with lysozyme as described in the Methods section of this paper.

Amino acid added	Specific activity of cell-wall protein (μc/g.) after 5 min.	Specific activity of cell-wall protein (μc/g.) after 30 min.	Specific activity of protoplast protein (μc/g.) after 5 min.	Specific activity of protoplast protein (μc/g.) after 30 min.
L-[^{14}C]Leucine	0·09	0·24	0·71	2·85
L-[^{14}C]Lysine	0·37	0·39	9·8	16·5
[1-^{14}C]Glycine	0·35	1·65	7·7	7·8
DL-[3-^{14}C]Phenylalanine	0·14	1·3	3·5	8·5

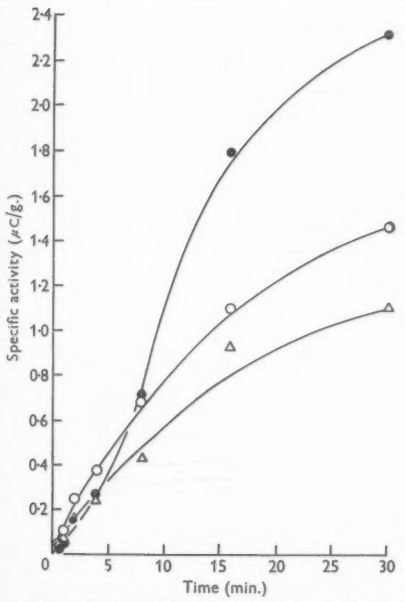

Fig. 1. Incorporation of [1-^{14}C]glycine into the protein of subcellular fractions of whole cells of *Bacillus megaterium*, incubated aerobically in an inorganic salts medium with glucose. The amino acid (10 μc) was added to 1 g. dry wt. of cells growing at 1 mg./ml. in this medium. Samples were taken at various times and analysed as described in the Methods section. ●, Cytoplasm; ○, cytoplasmic membrane; △, EDTA digest.

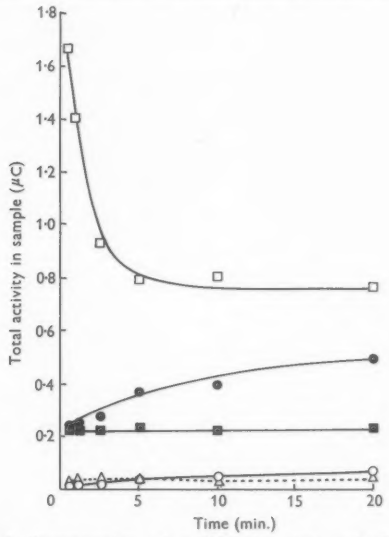

Fig. 2. Incorporation of L-[^{14}C]lysine into subcellular fractions of whole cells of *Bacillus megaterium*, incubated aerobically in an inorganic salts medium with glucose. The amino acid (12 μc) was added to 2 g. (dry wt.) of cells growing at 1 mg./ml. in the medium. Samples (333·3 ml. each) were taken at various times and analysed as described in the text. □, Cell wall; ●, cytoplasm; ■, non-protein from cytoplasm; △, EDTA digest; ○, cytoplasmic membrane.

The proteins of the EDTA digests had, in general, a lower specific radioactivity than either of the other fractions, except at the very shortest periods of incubation when they had slightly higher specific radioactivities than the cytoplasmic proteins. Similar results were obtained with other labelled amino acids, and it was usually unnecessary to separate cell-wall protein (or polypeptide material) from the cytoplasmic proteins, as most of it was removed during the precipitation and washing procedures, and it did not greatly affect the observed specific activity of the cytoplasmic protein.

However, in some experiments the curves for the specific radioactivities of the different fractions were closer than in others, particularly if the lysozyme-digestion procedure was performed at a higher temperature than 2° or for a prolonged period. It seemed likely that an unknown amount of equilibration of radioactive protein between the

various fractions was taking place as long as the cells remained intact.

At the same time the rate of incorporation of L-[^{14}C]lysine into the various cellular fractions of the organism was studied as before, but the fractions were dried at 80° and the total activity present in each fraction, including the non-protein material, was determined. The results are shown in Fig. 2. A very large proportion of the added amino acid was fixed in the cell wall and the bulk of the remainder was found in the cytoplasmic fraction. Two further points of interest were: first, the amount of label in the membrane and EDTA digests could at all times be accounted for almost entirely by the labelled protein present and, secondly, that the total radioactivity of the non-protein material present in the cytoplasm remained constant at all times of incubation from 30 sec. to

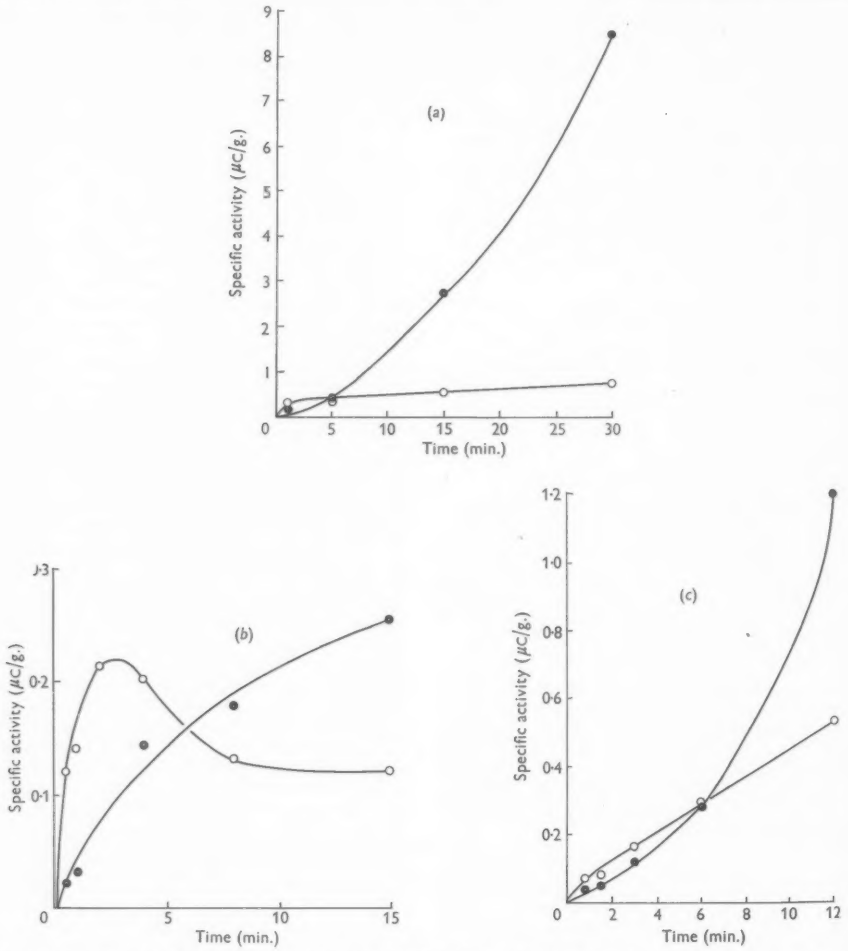

Fig. 3. Incorporation of [^{14}C]amino acids into the proteins of the membrane and cytoplasm after incubation of protoplasts of *Bacillus megaterium* in a medium containing 0·5 M-KH_2PO_4, other inorganic salts and glucose. In each case 10 μc of labelled amino acid was added to protoplasts from 1·5 g. (dry wt.) of whole cells growing at 1 mg./ml., and samples were taken and analysed as described in the Methods section. (*a*) Incorporation of [1-^{14}C]glycine; (*b*) incorporation of L-[^{14}C]lysine; (*c*) incorporation of L-[^{14}C]valine. ●, Cytoplasm; ○, cytoplasmic membrane.

20 min. These last figures were obtained by difference; after assay of the total activity, the dried cytoplasm was extracted with cold trichloroacetic acid and the radioactivity of the protein in the residue determined after the removal of lipid and nucleic acid material by standard methods.

The enormous reservoir of radioactive material within the cell walls would obviously make it difficult to interpret any experiments designed to follow the transfer of radioactive material between fractions during the course of protein synthesis. This led us to turn our attention to studies on the protoplast system, which had the twofold advantage of eliminating the cell wall and reducing the length of time required to fractionate the cellular components.

Experiments with protoplasts. In initial experiments, the great fragility of the protoplasts, combined with the necessity to maintain them under strictly aerobic conditions, made it difficult to obtain preparations that yielded consistent results. Conditions (described in the Methods section) were eventually established that gave satisfactorily reproducible results with our strain. When time-studies of the incorporation of labelled amino acids were carried out, it was found that the specific radioactivities of membrane and cytoplasmic fractions showed much greater differences than in many of the experiments carried out with whole cells. The membrane-protein fractions were again those most highly labelled at early times, but, after 5–7 min., the proteins of the cytoplasm attained higher specific radioactivities. Typical results with different ^{14}C-labelled amino acids are shown in Fig. 3. The differences between the three sets of curves probably reflect differences in the degree of damage to the protoplasts and not differences in the incorporation behaviour of the individual amino acids.

Experiments with broken protoplasts and isolated membranes. When protoplasts were disintegrated by rapid shaking in the Mickle tissue disintegrator and then incubated in the usual protoplast-incorporation medium, it was found that ^{14}C-labelled amino acids were taken up into the membrane protein at rates comparable with those observed in intact protoplasts (Fig. 4). Very little radioactivity was found in the cytoplasmic protein, however. This result was largely repeatable although in one subsequent experiment the cytoplasmic proteins reached a specific radioactivity of 25 % of that of the membrane protein after incubation for 30 min.

Much the same degree of incorporation of ^{14}C-labelled amino acids into membrane proteins was found if the membranes were separated from the cytoplasm by sedimentation and resuspended in 'C' solution with 1 % added glucose before carrying out the incubation (Fig. 5). The presence of traces of cytoplasm was not necessary because membranes twice washed with buffer were as active as membranes used immediately after sedimentation. The method used for breaking the protoplasts was also unimportant, as it was found that membranes prepared from protoplasts lysed by diluting the medium three times with water incorporated labelled amino acids into protein just as well as those obtained by breaking protoplasts in the Mickle disintegrator. Finally, full activity of the system was retained if the protoplasts were lysed and the membranes collected and used after storage for 2 days at 2° (Table 2).

The linear incorporation of labelled amino acids into membrane protein was found to continue for at least 2 hr. with L-[^{14}C]valine (Fig. 5). In other experiments, incubations were not continued for such a long period but the uptake was linear until the end of the experiment in all.

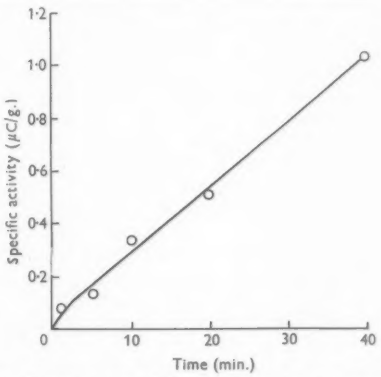

Fig. 4. Incorporation of [1-^{14}C]glycine into the protein of the membrane fraction of disrupted proplasts suspended at a density of 1 mg./ml. in a medium containing 0·5 M-KH_2PO_4, other inorganic salts and glucose. The amino acid (10 μC) was added to membranes derived from 1·5 g. (dry wt.) of whole cells. The disruption and subsequent analysis are described in the Methods section.

Table 2. *Incorporation of* [1-^{14}C]*glycine into membranes prepared from lysed protoplasts stored at 2° for varying lengths of time*

Incorporation of 2 μC of labelled amino acid was carried out for 30 min.; 100 mg. of membranes was used in each case. Other experimental conditions were as described in the text.

Time of storage of lysed protoplasts (days)	Incorporation of labelled amino acids into membrane protein (μC/g.)
0	8
1	11
2	7

The action of deoxyribonuclease on the system was also examined. Membranes were incubated with the enzyme before and after incubation with ^{14}C-labelled amino acid and the results are summarized in Table 3. These treatments had little effect although in both a small amount of protein was solubilized. However, prolonged incubation of the membranes at 37° with or without deoxyribonuclease did considerably reduce the rate at which amino acids were incorporated into the protein of the membranes.

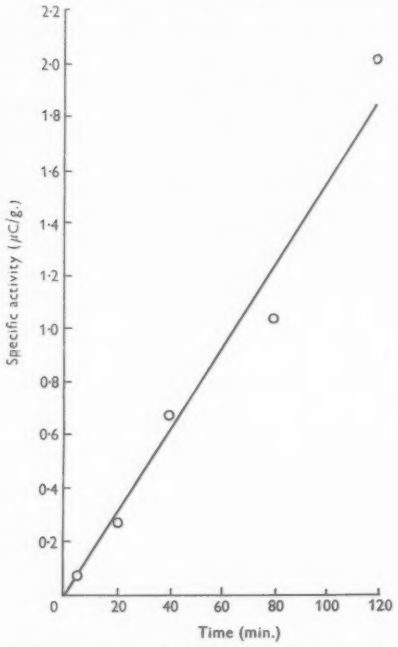

Fig. 5. Incorporation of L-[^{14}C]valine into the proteins of the membrane fraction isolated from disrupted protoplasts of *Bacillus megaterium*. The amino acid (10 μc) was added to membranes derived from 1·5 g. of whole cells suspended at 1 mg./ml. in a medium containing inorganic salts and glucose. The preparation of the fraction and subsequent analysis of the samples taken at various times is described in the Methods section.

Transfer of labelled protein from membranes to cytoplasm. At the same time, repeated attempts were made to obtain incorporation of ^{14}C-labelled amino acids into the proteins of isolated cytoplasm, diluted by the medium in which the protoplasts were broken. A small incorporation was observed, never more than 10% of the incorporation into isolated membranes and usually much less. The incorporation was not enhanced by including adenosine triphosphate, fructose 1:6-diphosphate or a complete mixture of amino acids in the medium. However, it was found that a very high degree of labelling of the cytoplasmic proteins could be achieved if they were incubated with washed membranes previously labelled *in vitro* as described above. Further, the proportion of radioactive label that could be transferred from membrane to cytoplasm was dependent upon the time for which the membranes had been incubated with the [^{14}C]amino acid. The results are summarized in Table 4, and it will be seen that no loss of labelled

Table 3. *Effect of digestion with deoxyribonuclease on the specific radioactivity of the membrane proteins*

Membranes (100 mg.) were treated at 37° for 1 hr. with deoxyribonuclease (DNA-ase; 50 μg./ml.) in 10 ml. of 'C' solution–phosphate medium.

The membranes were then sedimented and either resuspended for incubation or treated with trichloroacetic acid. Incubations were carried out with 2 μc of [1-^{14}C]-glycine as described in the text.

Treatment with DNA-ase before incubating membranes for 2 min. with labelled amino acid		Treatment with DNA-ase after incubating membranes for 30 min. with labelled amino acid	
DNA-ase	Specific activity of protein (μc/g.)	DNA-ase	Specific activity of protein (μc/g.)
+	0·36	+	3·9
−	0·38	−	4·1

Table 4. *Transfer of a labelled protein fraction from membranes labelled* in vitro *with* [1-^{14}C]*glycine to the protein fraction of unlabelled cytoplasm*

In each case, the membranes (approx. dry wt. 42 mg.) were incubated with cytoplasm (approx. dry wt. 93 mg.) for 5 min. All incubations were carried out aerobically at 30°.

Time of incubation of membranes with [1-^{14}C]glycine (min.)	Initial total radioactivity of membrane proteins (μmc)	Radioactivity of membrane proteins after incubation with cytoplasm (μmc)	Final radioactivity of cytoplasmic proteins (μmc)	Transfer (%)
1·5	2·7	1·1	1·6	59
10	22	15	8	36
30	51	39	10	20

material from the combined protein fractions occurs, and that the greatest percentage transfer was obtained with membranes that had been allowed to incorporate for the shortest time.

An attempt was made to determine the rate at which labelled protein is transferred from the membrane to the cytoplasm, membranes which had been pre-incubated for 20 min. with [^{14}C]-amino acid being used. It was concluded that this is a very rapid process as the transfer was found to be complete in 1 min., the smallest interval measured. On the other hand, very little radio-activity (never more than 5%) was lost from the protein fraction of previously labelled membranes when they were incubated in buffer alone for 30 min.

Analyses. The nucleic acid contents of whole cells, membranes and cytoplasm as determined by the methods of Cerriotti (1952, 1955) are given in Table 5. The values obtained differ from those given by Weibull (1953*b*), but his preparative procedures differed in that he prepared protoplasts in a sucrose medium and, probably more important, subsequently treated them with deoxyribonuclease before examining membranes and cytoplasm.

Crude nucleic acid isolated from the cytoplasm directly by precipitation of the proteins with sodium dodecyl sulphate was found by base analysis to consist of 89% of RNA and 11% of DNA, in good agreement with the results given by the colour reactions. But the treatment of finely divided membranes with sodium dodecyl sulphate liberated nucleic acid into solution that was shown, after purification, to contain only traces of RNA. Weibull's membranes contained very little RNA, but the bulk of the RNA could be extracted from the cytoplasm by centrifuging for 2½ hr. at 105 000 *g* and sedimentation studies showed that there were three macromolecular components present with sedimentation coefficients of 42, 26 and 3s. In our preparations sedimentation analysis of the cytoplasm, performed by Dr K. V. Shooter of this Institute, showed that there were three macromolecular components with sedimentation coefficients of 13, 6 and 3s and at least 80% of the material was present in the 3s component.

Table 5. *Composition of whole cells, cytoplasmic membranes and cytoplasm of* Bacillus megaterium, *prepared as described in the text*

	Dry wt. of whole cells (%)	DNA (%)	RNA (%)
Whole cells	100	1·5	8·0
Cytoplasmic membranes	29	2·8	3·7
Cytoplasm	43	1·3	9·9

The cytoplasm, as prepared by us, thus contains no particles comparable with microsomes. In fact it can be estimated that the 3s component has a molecular weight of about 30 000.

DISCUSSION

The initial experiments with whole cells show that although the cell wall contains large quantities of free amino acids, it plays very little part in, at least, the initial processes of protein synthesis in the remainder of the cell. It may act as a reservoir of amino acids and provide a constant supply to the organism. This could account for the consistency of the amount of unincorporated amino acids found in the cytoplasm which are either in the free state or in the form of simple derivatives.

In the whole-cell experiments the time required for the digestion by lysozyme probably did result in some equilibration between the labelled protein of the membrane and cytoplasmic fractions, because, in the more rapidly fractionated protoplast experiments, the differences in the labelling of these fractions are much more marked. Although all experiments show an initially greater labelling of the membrane, which is later exceeded by the cytoplasm, individual differences observed in experiments are difficult to interpret. It is, however, not easy to be certain that, in any one experiment, all the protoplasts are active or even that all of them are intact.

However, the feature common to all these curves strongly suggests that the initial incorporation of amino acid into protein occurs in the membrane fraction and is followed by transfer to the cytoplasm. The further rise, usually observed after 6 min., in the specific activity of the proteins of the cytoplasmic membranes is then explicable if protein, initially formed in the membranes, can be used, possibly after further modification for structural or enzymic purposes in the membranes themselves as well as in the cytoplasm.

Our conclusions are supported by the results of the experiments in which the isolated membranes were labelled *in vitro*. It is to be noted that, in many cases, a similar level of labelling was observed with isolated membranes as with membranes from whole protoplasts. That the cytoplasm does not play a vital part in this process is borne out by the fact that its complete removal by washing did not prevent incorporation. A suggestion that the cytoplasmic membranes of *B. megaterium* may be a site of protein synthesis has also been made elsewhere (McQuillen, 1956).

The actual amount of amino acid incorporated into the proteins is sufficient to show that the mechanism studied cannot be dismissed as an insignificant process. Thus in one experiment an

incorporation of 50 μg. of [1-^{14}C]glycine into 50 mg. of membrane proteins was observed; the labelling was not reduced by dialysing the membranes against four changes of water at 1° before adding trichloroacetic acid. This amount represents an incorporation equivalent to about 2% of the pre-existing glycine residues.

The length of time for which the membranes will continue to incorporate ^{14}C-labelled amino acids into their protein fraction is also remarkable, the system being much more active in this respect than other systems *in vitro* already described. The part played by the cytoplasm in the intact cell is not clear. It appears to be the final repository of the bulk of the label and thus may be the eventual receptacle of many newly formed proteins. The transfer of labelled protein from membranes to cytoplasm removed previously is a very rapid process and occurs without loss of labelled protein. This suggests that protein is being transferred rather than amino acids or simple derivatives and is in itself indirect confirmation that the processes studied *in vitro* are closely allied to protein synthesis.

Much less radioactivity appeared in the cytoplasmic-protein fraction in the broken-protoplast experiments than in the transfer experiments. The reason for this discrepancy is not clear but could be explained by production of a soluble transfer inhibitor in the cytoplasmic membranes which is excreted into the medium in the whole-protoplast experiments and is also kept from contact with the cytoplasm by an intervening sedimentation in the transfer experiments.

We can conclude that these experiments give an indication that the main site of protein synthesis in *B. megaterium* is the cytoplasmic membrane. It must be remembered that the 'membrane fraction' obtained in these experiments includes not only the actual protoplast membrane, but also the lipid granules and part, at any rate, of the DNA gel. However, the cytoplasmic membranes themselves constitute the bulk (75%) of the fraction, and there seems little doubt that they provide the sites for the incorporation of labelled amino acids. Thus our experiments with deoxyribonuclease make it seem unlikely that DNA or protein associated with it plays an important role in the incorporation process. This is further borne out by the experiments with EDTA which showed, although the results obtained were rather variable, that protein solubilized with the bulk of the DNA, and possibly associated with it, was not as readily labelled as the residual protein. Besides, a large proportion of the cellular DNA occurs in the cytoplasmic fraction, and the material of the original DNA-containing gel is probably distributed almost equally between our membrane and cytoplasmic fractions. It seems most unlikely that the lipid granules play any direct part in protein synthesis: these also are digested by EDTA. The 'small-particle' fraction obtained by Weibull (1953*b*) is absent from our cytoplasm. Whether this arises from the dissolution of the gel by Weibull's deoxyribonuclease or the cytoplasmic membranes is an open question; but Mitchell & Moyle (1956) have shown that the 'small-particle' fraction of *Staphylococcus aureus* probably arises as a result of the disintegration of the delicate cytoplasmic membrane of that organism. Methods of fractionating the cytoplasmic membranes are at present being studied in more detail.

SUMMARY

1. The incorporation of ^{14}C-labelled amino acids into protein has been studied in whole cells of *Bacillus megaterium* and also in protoplasts and various subcellular fractions derived from the same organism.

2. The cell wall does not incorporate any of the amino acids studied into its 'protein' to any great extent, but it absorbs a large quantity of free amino acid.

3. Initially, labelled amino acids are incorporated most rapidly into the protein derived from the cytoplasmic membranes of whole cells and protoplasts incubated in growth media. At later times the cytoplasmic proteins attain higher specific activities. At all times the level of the non-protein radioactivity is highest in the cytoplasm.

4. Isolated cytoplasmic membranes can incorporate ^{14}C-labelled amino acids into their protein fractions at rates comparable with those observed in the membranes of whole protoplasts: the incorporation is linear for at least 2 hr. In comparison, isolated cytoplasm incorporated very small amounts of amino acid into protein.

5. If membranes labelled in this way are incubated with non-labelled cytoplasm, a considerable proportion of the radioactivity is rapidly transferred from the membrane protein to the cytoplasmic protein.

6. It is concluded that the initial stages of protein synthesis in *B. megaterium* take place at sites on or closely associated with the cytoplasmic membrane.

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The Metabolic Stability of the Nucleic Acids in Cultures of a Pure Strain of Mammalian Cells

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Although in recent years the metabolism of the nucleic acids has been the subject of many experiments with isotopic-tracer techniques, our knowledge of the relative rates at which deoxyribonucleic acid (DNA), nuclear ribonucleic acid (nRNA) and cytoplasmic ribonucleic acid (cRNA) are synthesized and broken down is still far from complete.

The present experiments were designed to provide answers to two particular questions: (1) What inferences about nucleic acid synthesis can be drawn from the manner in which [^{14}C]formate is incorporated into the purines and pyrimidines of DNA, nRNA and cRNA? (2) To what extent are DNA, nRNA and cRNA, once synthesized, metabolically stable in animal cells?

Information of this kind cannot readily be obtained from the intact animal or from tissue slices surviving *in vitro*. Use was therefore made of a pure strain of mammalian cells growing in tissue culture. Brief preliminary reports of some of the results obtained have already been published (Thomson, Paul & Davidson, 1956; Thomson & Paul, 1957).

METHODS

Biological

The cells used in these experiments were adult mouse subcutaneous fibroblasts NCTC strain L, clone 929 (Sanford, Earle & Likely, 1948), hereafter referred to as L cells. These cells were grown directly on the floor of large conical culture flasks having an area of about 140 cm.^{2}. The medium in which they were normally maintained was composed of 20 parts of horse serum, 10 parts of chick-embryo extract and 70 parts of Hanks's balanced salt solution (Hanks & Wallace, 1949). It also contained 50 units of sodium penicillin G/ml. but no other antibiotics.

In most of the experiments to be described, similar vessels were used. In each vessel was placed a glass-covered magnet so that the cells could readily be suspended by placing the culture vessel on a magnetic stirrer. This procedure proved less damaging to the cells than treatment with trypsin. In the conditions described the cells usually reached a maximum population of between 50 and 80 millions/flask, at which level the population became stationary. In all experiments the population was kept near the maximum, the cultures being halved at each transfer and allowed to grow to the maximum once more. The medium, 60 ml./flask, was completely renewed twice a week and on one of these occasions the cells were transferred.

In this way, a slow rate of growth was maintained and the cells remained healthy throughout. In some experiments, where it was necessary to exclude extracellular pools of purines and pyrimidines, the medium used was Eagle's medium (Eagle, 1955) supplemented with 30% (v/v) of dialysed horse serum. Also, in some experiments where the inocula of cells were much smaller than those described above, smaller vessels were used, e.g. 25 ml. Erlenmeyer flasks.

At the commencement of an experiment a suspension of cells was obtained by treating stock cultures of cells with

0·5% (w/v) trypsin (Difco 1/250). After being centrifuged and resuspended in growth-promoting medium, the cells were counted in a haemocytometer chamber and a suitable amount was inoculated into each experimental flask.

The isotope was introduced into the medium as sodium [^{14}C]formate, usually at a final concentration of 2 μc/ml. In two of the long-term experiments, where it was desired to achieve a high degree of labelling initially, this concentration was doubled. In these experiments also 5-amino-4-imidazole carboxamide (AICA) riboside (California Foundation for Biochemical Research) was added in a concentration of 40 μg./ml. during the incorporation phase in order to promote labelling of the purines, as described later.

In some experiments in which retention of isotope in nucleic acids and acid-soluble bases was measured, unlabelled sodium formate was added to the medium, after the withdrawal of the labelled formate, to give a concentration of 22 μg./ml., this being the same concentration of formate as was present during incorporation.

As samples were harvested, they were either fractionated immediately or stored at $-70°$ until it was convenient to carry out the analyses. By gently homogenizing in 0·1 M-citric acid the cells were disrupted. At this point the volume of suspension was carefully noted and a small sample taken for counting of nuclei. For this purpose the nuclei were further diluted with a solution of 0·1% (w/v) crystal violet in 0·1 M-citric acid and transferred to a Fuchs–Rosenthal chamber. Two independent observers each performed a minimum of two counts of each specimen, so that in each case about 1000 nuclei were counted (see Sanford *et al.* 1951). The remaining suspension of disrupted cells was then further fractionated as described below.

Chemical

The chemical treatment of the cells for the separation of nuclear and cytoplasmic fractions and for the determination of the specific activities of the bases in DNA, nRNA and cRNA and of the bases in the acid-soluble nucleotide fraction was carried out as described by Smellie, Thomson & Davidson (1958). cRNA was determined by ultraviolet-absorption measurement (Smellie *et al.* 1958) on the solution made by extracting the dried lipid-free cytoplasmic powder with hot 0·5 N-perchloric acid. A small portion of the nuclear suspension was used for determination of DNA by the method of Schmidt & Thannhauser (1945) as modified by Davidson & Leslie (1951), phosphorus being estimated by the method of Griswold, Humoller & McIntyre (1951).

The total activities of DNA and cRNA were calculated on the assumption that the bases were present in equimolar proportions.

RESULTS

Table 1 shows the pattern of incorporation of [^{14}C]formate into the nucleic acid bases of L cells growing in embryo extract–horse serum medium. As might be predicted on the basis of current theories of purine and pyrimidine biosynthesis (Buchanan *et al.* 1957), the isotope was incorporated into the adenine and guanine of DNA, nRNA and cRNA and into the thymine of DNA. The nucleic acid pyrimidines other than thymine showed no activity. The observation that the DNA thymine was much more heavily labelled than the

Table 1. *Pattern of incorporation of* [^{14}C]*formate into the nucleic acid bases of L cells grown for* 2 *days in* (*a*) *horse serum–embryo extract* (*control medium*), (*b*) *control medium plus liver extract, and* (*c*) *control medium plus* 5*-amino-*4*-imidazole carboxamide riboside*

Results are expressed in terms of relative specific activities with the value for thymine as 100. The actual specific activity of the thymine in all three cases was of the order of 100 000.

Medium	Base analysed	Relative specific activities (counts/min./μmole) DNA	nRNA	cRNA
(*a*) Control	Adenine	11	18	17
	Guanine	14	23	13
	Thymine	100	—	—
(*b*) Control + liver extract	Adenine	145	238	205
	Guanine	168	326	—
	Thymine	100	—	—
(*c*) Control + 5-amino-4-imidazole carboxamide riboside	Adenine	122	177	160
	Guanine	148	232	188
	Thymine	100	—	—

Table 2. *Retention of* ^{14}C *in deoxyribonucleic acid bases of L cells growing in horse serum–embryo extract medium*

	Total activity (counts/min.) Adenine	Guanine	Thymine
Cells grown for 24 hr. in medium containing [^{14}C]formate	350	1284	91 800
Cells grown for a further 96 hr. in non-radioactive medium	580	1060	108 300

DNA purines is in accord with similar observations made with other tissues *in vitro* (Smellie, Thomson, Goutier & Davidson, 1956). The relatively heavy labelling of thymine might be due either to true DNA synthesis, the poor incorporation into nucleic acid purines being attributable to utilization of existing stocks of unlabelled purines for nucleic acid synthesis, or to a rapid exchange reaction between the DNA thymine and the [^{14}C]formate of the medium. A decision between these alternatives has been made by growing the labelled cells for several days in non-radioactive medium containing inactive formate (Table 2). If the high labelling of the thymine had been due to an exchange reaction, the isotope would have been rapidly released in the inactive medium; on the contrary, the activity of the thymine showed an appreciable increase, presumably as a result of incorporation of labelled precursors formed while the cells were still in the active medium. Consequently, it can be concluded that the incorporation of [^{14}C]formate into thymine is due to true DNA synthesis.

In order to determine whether the behaviour of the cultured cells resembles the known behaviour of normal and neoplastic tissues *in vivo* and *in vitro*, an examination was made of the effect on L cells of mouse-liver extract, which has been shown to stimulate incorporation of [^{14}C]formate *in vitro* into the nucleic acid and acid-soluble purines of several rapidly growing tissues, notably rabbit bone marrow and the Ehrlich ascites carcinoma of the mouse (Smellie *et al.* 1956). The extract produced a pronounced increase in incorporation of [^{14}C]formate into the acid-soluble adenine and nucleic acid purines of the L cells (Table 1) without causing any appreciable difference in incorporation into DNA thymine. The liver extract therefore appeared to have produced a great increase in purine synthesis without affecting the rate of formation of DNA, presumably by supplying either intermediates involved in purine synthesis or the enzymes responsible for their formation. Support for this view is obtained by the observation that the addition of 5-amino-4-imidazole carboxamide riboside, the phosphate ester of which is an intermediate in purine synthesis (Buchanan *et al.* 1957), greatly increases incorporation of [^{14}C]formate into the nucleic acid purines of L cells (Table 1) but not into the DNA thymine. This observation also is in accord with similar results obtained in ascites-tumour and other cells (Smellie *et al.* 1958), and has been made use of in later experiments with L cells in order to obtain good initial labelling of the purines in studies of subsequent isotope loss.

If incorporation of [^{14}C]formate into DNA thymine occurs only in the course of DNA synthesis and not as a result of an exchange reaction, it follows that the disproportionately low incorporation of [^{14}C]formate into DNA purines found in most experiments with L cells must be due, as suggested above, to the utilization by the cells of unlabelled purines from the medium for DNA synthesis. This question was investigated by growing L cells in Eagle's semi-synthetic medium (supplemented with dialysed serum), which contains no purines. Under these conditions the cells incorporated [^{14}C]formate extensively into their nucleic acid purines so that DNA adenine and guanine developed a specific activity twice as great as that of DNA thymine (Table 3). This is, of course, in accordance with the theory that formate contributes two carbon atoms to each molecule of purine and one to each molecule of thymine.

Table 3. *Incorporation of* [^{14}C]*formate into nucleic acid bases and acid-soluble bases of L cells growing for* 48 *hr. in Eagle's medium with dialysed serum and the supplements shown*

Supplement added to medium	Base analysed	Counts/min./μmole of base			
		DNA	nRNA	cRNA	Acid-soluble bases
1. 0	Adenine	81 600	306 000	121 000	411 000
	Guanine	94 800	236 400	107 500	—
	Thymidine	46 000	—	—	—
2. Adenosine (0·2 μmole/ml.)	Adenine	1 100	20	1 720	7 510
	Guanine	1 700	2 720	1 680	—
	Thymidine	34 800	—	—	—
3. Guanosine (0·2 μmole/ml.)	Adenine	42 400	110 200	66 700	151 600
	Guanine	9 780	18 440	10 920	—
	Thymidine	44 400	—	—	—
4. Thymidine (0·2 μmole/ml.)	Adenine	66 800	243 500	102 700	371 500
	Guanine	60 800	175 700	109 700	—
	Thymidine	3 040	—	—	—
5. Azaserine (10 μg./ml.)	Adenine	28	2 980	1 080	6 160
	Guanine	0	1 840	1 021	—
	Thymidine	17 500	—	—	—

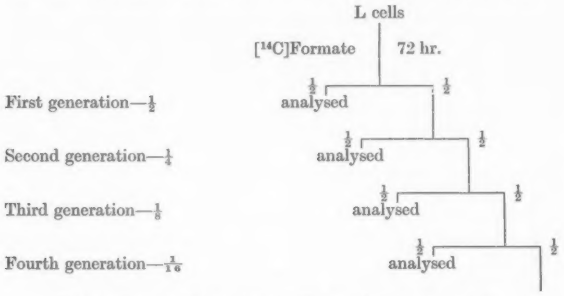

Fig. 1. Design of experiment. For explanation see text.

Addition of adenosine markedly diminished incorporation of [^{14}C]formate into nucleic acid purines and acid-soluble adenine, without greatly affecting incorporation into DNA thymine. Addition of guanosine produced similar but less-extensive effects. Conversely, addition of thymidine diminished incorporation into DNA thymine without greatly affecting incorporation into acid-soluble adenine or nucleic acid purines. Clearly therefore the cells are capable both of synthesizing their own purines and pyrimidines and of utilizing purines and pyrimidines present as nucleosides in the medium.

Table 3 also shows the effects of azaserine [$\overset{-}{N}:\overset{+}{N}:CH\cdot CO\cdot O\cdot CH_2\cdot CH(NH_2)\cdot CO_2H$], which is known to inhibit purine synthesis by inhibiting the conversion of formyl glycinamide ribotide into formyl glycinamidine ribotide (Levenberg, Melnick & Buchanan, 1957). As might be expected, azaserine drastically inhibited incorporation into acid-soluble adenine and nucleic acid purines, but incorporation into DNA thymine, although depressed, was still sufficiently pronounced to indicate substantial DNA synthesis. Therefore even when the cells are growing in a purine-free medium and have been prevented from synthesizing purines for themselves, they still continue to synthesize DNA for some time at the expense of their existing stocks of purines in a manner analogous to that found in the Ehrlich ascites carcinoma *in vitro* (Smellie *et al.* 1958).

These experiments were all concerned with the biosynthesis of the nucleic acids. To investigate their metabolic stability it was necessary to adopt a different experimental plan (Fig. 1). L cells were grown in embryo extract–horse serum medium containing 5-amino-4-imidazole carboxamide riboside and [^{14}C]formate until the nucleic acids were heavily labelled in the purines and thymine. These labelled cells were transferred to non-radioactive medium, in which they were grown at a slow rate for eight or nine generations, samples being taken

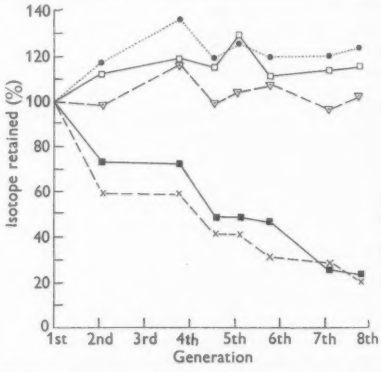

Fig. 2. Total activity of the nucleic acid bases in the DNA and cytoplasmic RNA of L cells grown for several generations in non-radioactive medium after initial labelling with [^{14}C]formate. All values are expressed as percentages of the initial values. ●, DNA guanine; □, DNA adenine; ▽, DNA thymine; ■, cRNA guanine; ×, cRNA adenine.

for analysis from time to time. By this means it was possible to determine the degree to which the ^{14}C initially incorporated was retained in the bases of DNA, nRNA and cRNA and hence to estimate their varying degrees of metabolic stability.

The plan of the first experiment of this type is shown in Fig. 1. Immediately on removal from the radioactive medium the culture was divided into two equal parts, one of which was analysed. The other was allowed to grow in non-radioactive medium for about one generation, i.e. until the cell number had approximately doubled. Then it in turn was divided into two equal parts, one of which was analysed while the other was allowed to grow for a generation in non-radioactive medium. This process was continued for eight generations. In such an experiment the first sample analysed is equivalent to half the original culture, the second

to one-quarter, the third to one-eighth and so on. From the amounts and specific activities of the DNA, nRNA and cRNA of each sample the degree to which the ^{14}C originally incorporated had been retained in each of the nucleic acid bases could be calculated.

The specific activities of the bases in this experiment (Table 4) showed a steady fall, the drop being more rapid in RNA bases than in DNA bases. It is important to note that the figures for nRNA and cRNA remained remarkably close to each other during the fall. On the other hand, a very different pattern is observed when the total activities of the bases of DNA and cRNA are calculated (Fig. 2). During the first two generations after the transfer to non-radioactive medium, the total amount of ^{14}C in DNA adenine, guanine and thymine tended to increase slightly, presumably because of the incorporation of precursors which had taken up ^{14}C while the cells were in radioactive medium. Thereafter the amount of ^{14}C retained by all three bases tended to remain steady or to fall slightly.

In striking contrast with the behaviour of the DNA, the amount of ^{14}C retained in cRNA adenine and guanine fell rapidly and continuously. The conclusion suggested by these results is that whereas DNA once formed may be metabolically stable or only very slowly broken down, some RNA is continually being broken down and resynthesized at a fairly rapid rate. These results have been confirmed by subsequent experiments of the same type.

Table 4 demonstrates that at the beginning of the experiment the acid-soluble adenine had a specific activity about twice as high as that of the nRNA and cRNA adenine. This fell very sharply during the first generation in non-radioactive medium but throughout the remainder of the experiment remained at a level only slightly lower than that of the nRNA and cRNA adenine.

The very high degree of retention of ^{14}C in the DNA bases in this experiment might not have been entirely due to the stability of the DNA molecule since it is quite possible that DNA broke down slowly to give labelled products, but that these were completely, or almost completely, utilized for the synthesis of new DNA, so that there was no overall loss of ^{14}C from the DNA of the culture as a whole. For kinetic reasons such reutilization of labelled breakdown products would be most extensive in rapidly growing tissues. In the present experiment the rate of growth was relatively slow with a mean generation time of 4–7 days. By this means reutilization of labelled-breakdown products could be reduced, but not completely eliminated. Theoretically, it could be reduced still further if the cells were flooded with unlabelled nucleic acid precursors such as nucleosides and nucleotides.

Table 4. *Specific activity of nucleic acid bases at each generation during the growth of ^{14}C-labelled L cells in non-radioactive medium*

Approx. generation no.	Cell population (millions)	Base analysed	Specific activities (counts/min./μmole) DNA	nRNA	cRNA	Acid-soluble bases
1	138	Adenine	182 000	192 000	200 000	446 000
		Guanine	149 200	144 000	183 400	—
		Thymine	77 000	—	—	—
2	288	Adenine	76 700	47 950	48 800	30 200
		Guanine	67 200	55 500	56 000	—
		Thymine	28 700	—	—	—
3	1 008	Adenine	30 800	12 460	18 700	10 360
		Guanine	29 625	19 900	20 400	—
		Thymine	12 920	—	—	—
4	1 840	Adenine	15 900	5 610	6 420	2 360
		Guanine	13 520	6 320	6 960	—
		Thymine	5 780	—	—	—
5	2 624	Adenine	11 800	3 415	4 480	2 470
		Guanine	9 495	4 000	4 740	—
		Thymine	3 970	—	—	—
6	4 352	Adenine	5 300	1 860	1 650	755
		Guanine	4 750	2 004	2 332	—
		Thymine	2 175	—	—	—
7	10 752	Adenine	2 200	560	628	462
		Guanine	1 940	650	543	—
		Thymine	800	—	—	—
8	17 920	Adenine	967	202	188	165
		Guanine	856	220	194	—
		Thymine	362	—	—	—

To investigate this point, a second experiment was set up to determine whether the retention of ^{14}C in DNA thymine and nucleic acid adenine was affected if the cells were supplied with unlabelled thymidine and adenosine. If the observed retention were due to absence of breakdown of DNA, it would obviously be unaffected by the presence of the thymidine and adenosine. On the other hand, if it were attributable to the reutilization of products of DNA breakdown for the synthesis of new DNA, the flooding of the cells with unlabelled thymidine would greatly dilute the labelled thymidylic acid and thymidine liberated by DNA breakdown and thus reduce the degree to which they were incorporated into new DNA. Addition of unlabelled adenosine might similarly diminish reutilization of any labelled deoxyadenylic acid or deoxyadenosine liberated by DNA breakdown.

The design of this experiment (Fig. 3) closely resembled that of its predecessor. As before, the cells were labelled by growing them in the presence of [^{14}C]formate and 5-amino-4-imidazole carboxamide riboside. Half of the culture was then taken for analysis and the remainder allowed to grow for about a generation in non-radioactive medium. Then it in turn was divided into two equal parts, one of which (equivalent to one-quarter of the original culture) was analysed. The other was allowed to grow for a further generation. The cells, which by this point had presumably utilized all the [^{14}C]formate which they had taken up, were then suspended in inactive medium and eight portions, each equal to 1/64 of the total culture and equivalent to 1/256 of the original culture, were dispensed into flasks. The cells in each flask were maintained for a further six generations: four flasks in ordinary non-radioactive medium; two in non-radioactive medium containing adenosine; two in non-radioactive medium containing thymidine. They were then analysed. The remainder of the culture (equivalent to 56/256 of the original culture) was analysed immediately. The degree of retention of ^{14}C in each of the nucleic acid bases was calculated as before.

The results are shown in Table 5. During the earlier part of the experiment covering approximately the first two generations in non-radioactive medium, retention of ^{14}C in the adenine, guanine and thymine of the DNA was almost complete. But in the latter part of the experiment, the degree of retention was dependent on the nature of the medium in which the cells were grown. Thus in

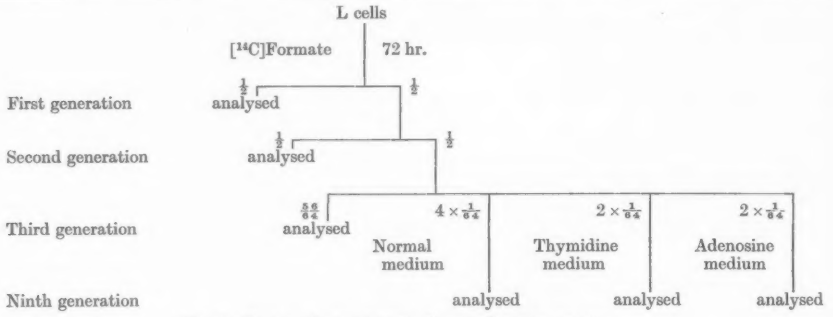

Fig. 3. Design of experiment. For explanation see text.

Table 5. *Percentage of initial radioactivity retained in bases of deoxyribonucleic acid and cytoplasmic ribonucleic acid of L cells growing in embryo extract–horse serum medium with and without addition of adenosine and thymidine*

Figures represent the means of several samples.

	DNA			cRNA	
	Adenine	Guanine	Thymine	Adenine	Guanine
Initial sample	100	100	100	100	100
Second sample	80·4	92·7	83·0	74·1	80·6
Third sample	83·1	93·9	93·1	47·2	51·5
Final sample (ordinary medium)	58·1	70·2	72·7	21·4	13·2
Final sample (ordinary medium + thymidine)	58·9	68·2	51·9	4·7	8·4
Final sample (ordinary medium + adenosine)	63·8	71·5	70·9	8·63	9·4

ordinary medium 60% of the ^{14}C initially incorporated into the DNA adenine and 70% of that initially incorporated into DNA guanine and thymine were retained at the end of the experiment. In the cells grown in the thymidine-containing medium the percentages of ^{14}C retained in DNA adenine and guanine were very similar to those found in ordinary medium. This suggests that the presence of thymidine had no effect in accelerating DNA breakdown. On the other hand, retention in DNA thymine was substantially lower (about 50% as opposed to about 70%).

It appears therefore that if the argument set out above is valid, the high degrees of retention of ^{14}C in DNA in this experiment and its predecessor were due in part to reincorporation of labelled breakdown products. The presence of adenosine in the medium had no apparent effect on retention of ^{14}C in DNA purines, possibly because the medium contains some purines so that the addition of more does not alter the situation greatly. As in the previous experiment, the purines of cRNA retained ^{14}C to a much smaller extent than did those of DNA.

The most important result of this experiment, namely that retention of ^{14}C in DNA thymine is depressed if unlabelled thymidine is present in the medium, implying as it does a measurable breakdown of DNA, appeared to merit confirmation. Similar results were obtained in a further experiment on the same lines.

DISCUSSION

It is clear from the results presented in Table 1 that L cells growing in culture present the same pattern of incorporation of [^{14}C]formate as is found with Ehrlich ascites carcinoma and certain other cells under conditions *in vitro* (Smellie *et al.* 1956, 1958). The most satisfactory explanation of the relatively low activities of the DNA adenine and guanine in comparison with thymine is that incorporation into the thymine is a measure of true DNA synthesis and that when preformed purines are available in the medium they are preferentially utilized for nucleic acid synthesis (Smellie *et al.* 1958). Confirmation of this view is obtained from the observation that in a purine-free medium incorporation into the purines is higher than that into thymine. Clearly the L cells can synthesize purines if the need arises, but they will utilize preformed adenosine, guanosine or thymidine if any of these nucleosides are provided (Table 3). Moreover, if purine synthesis is blocked by azaserine nucleic acid synthesis can still take place, albeit on a much reduced scale, presumably at the expense of limited existing stocks of purines in the cells.

It is also clear from Table 3 that adenosine may act as a precursor of nucleic acid guanine and guanosine of nucleic acid adenine, although our findings are in agreement with those of Brown & Balis (1957) in showing that the conversion of guanosine into adenosine is not so readily achieved as the reverse reaction.

The results of the retention experiments reported here indicate quite clearly that DNA is metabolically much more stable than nRNA or cRNA and thus confirm the generally accepted opinion on this question (see Davidson, 1957). However, the observations might conceivably be the result of the release from dead or dying cells of nucleic acid breakdown products which are subsequently reutilized from the culture medium. An explanation along these lines seems rather unlikely since it would be necessary to assume that the breakdown products of DNA were more extensively reutilized than those from RNA. Furthermore, the cells were grown in a monolayer under a thin layer of fluid and their mean generation time was of the order of 3 or 4 days. It is therefore probable that breakdown products of dying cells would readily diffuse throughout the medium. Since the volume of the medium was never less than 250 times the volume of the cells and since it was totally changed at least 15 times during the experiment, it is probable that any labelled nucleic acid breakdown products released by dying cells would be so extensively diluted as to prevent appreciable reincorporation taking place from the medium.

The fact that the activities of the three labelled bases in DNA keep in parallel with each other argues against the possibility that loss of isotope is due merely to an exchange reaction similar to the reversible exchange of thymine for 5-bromouracil, which has been observed in the DNA of non-dividing cells of *Escherichia coli* by Zamenhof, Reiner, de Giovanni & Rich (1956). In any case, the results reported in Table 2 provide additional evidence for the absence of such exchange reactions in our system.

Nevertheless, a change in the ratios of the activities of the three labelled bases in DNA was obtained by introducing a large pool of thymidine to the system (Table 5). Its effect in diluting the activity of DNA thymine indicates that there may be some degradation of DNA within the cell with subsequent reutilization of the breakdown products.

The metabolic stability of DNA has been the subject of several other investigations. Brues, Tracy & Cohn (1944) have shown that ^{32}P incorporated into the DNA of rat liver during the regeneration after partial hepatectomy is extensively retained for 2 weeks after the operation. Similar results are reported by Barton (1954). Hecht & Potter (1956) and Takagi, Hecht & Potter (1956) have also carried out similar experiments

with [6-^{14}C]orotic acid and found that isotope incorporated into DNA of the liver during regeneration is almost completely retained during the 50–100 days after the operation. Fresco, Bendich & Russell (1955) labelled the DNA of rat liver regenerating after partial hepatectomy with [1:3-$^{15}N_2$]adenine and [2-^{14}C]glycine and observed the disappearance of ^{15}N and ^{14}C during the 3 months after the thirtieth post-operative day. They found a very small loss of activity which could be accounted for by an equivalent small gain in liver weight during the experimental period. Extensive retention of isotope has also been found in the DNA of the livers of young growing rats (Swick, Koch & Handa, 1956; Kihara, Amano & Sibatani, 1956; Takagi *et al.* 1956). It should, however, be noted that in several of the experiments on liver there appeared to be some breakdown comparable with that which we have found in the present studies (Hecht & Potter, 1956; Takagi *et al.* 1956; Swick *et al.* 1956). For example, Swick *et al.* (1956) noted a 50% loss of DNA activity between the third and sixth weeks after incorporation, which they attributed to the death of short-lived cells.

Our present finding that there is a more rapid loss of isotope from nRNA and cRNA than from DNA is also in agreement with the results obtained from rat liver (Brues *et al.* 1944; Furst & Brown, 1951; Hecht & Potter, 1956; Takagi *et al.* 1956; Kihara *et al.* 1956; Swick *et al.* 1956). The fact that the activities of the nRNA and cRNA decrease in parallel is in agreement with the results obtained from regenerating rat liver by Takagi *et al.* (1956), who suggested that both might be in reversible exchange with a pool of acid-soluble precursors which is at least partly common to both. The observation that in the present experiments the specific activity of the acid-soluble adenine was of the same order as that of the nRNA and cRNA adenines supports this suggestion.

The observations summarized in Fig. 2 demonstrate that RNA is broken down in the living cells, and the fact that some breakdown products can be utilized is evident from Table 3. At the same time there is a tendency for the cells to retain even small molecules, as shown by the fairly high activity retained in the acid-soluble adenine throughout the experiments illustrated in Table 4. This suggests that, when RNA is degraded, the breakdown products are reincorporated into new RNA. It is possible that similar kinetic considerations may apply to all or part of the DNA. If the nucleic acids are partially broken down and the products subsequently reutilized *within the cell*, it is clear that any leakage to the medium will be greatest if the cells are grown slowly over a long period of time.

The retention of isotope in the nucleic acids of L cells in tissue culture has also been investigated by Siminovitch & Graham (1956*a*), Healy, Siminovitch, Parker & Graham (1956) and Graham & Siminovitch (1957), who found that $^{32}PO_4^{3-}$ ion incorporated into the nucleic acids of cells growing logarithmically was completely retained in both DNA and RNA. This result is in marked contrast with the loss of activity from RNA in the present experiments and in the experiments on rat liver cited above. It is, however, in agreement with similar observations on logarithmically growing *E. coli* (Hershey, 1954; Fujisawa & Sibatani, 1954; Siminovitch & Graham, 1956*b*). It would therefore appear that isotope may be retained in rapidly growing cells to a much greater extent than in cells growing more slowly.

This retention of label in both RNA and DNA during logarithmic growth is presumably due to the inevitably rapid incorporation of all precursors into cell constituents, with the result that some constituents, such as RNA, are increasing so quickly that breakdown products, if any, would immediately be reincorporated and no demonstration of turnover would be possible. The degree to which such reincorporation of breakdown products takes place will obviously be greatest where the rate of synthesis is much greater than that of breakdown. The situation in a system such as we employ, in which the cells grow more slowly, is quite otherwise and the difference between the results reported in this paper and those reported by Siminovitch & Graham (1956*a*), by Healy *et al.* (1956) and by Graham & Siminovitch (1957) can be adequately explained as being due to the differences in the kinetics of the two experiments.

In the system described in the foregoing experiments the reactions can be summarized thus

$$A \underset{3}{\overset{1}{\rightleftharpoons}} B \underset{4}{\overset{2}{\rightleftharpoons}} C,$$

where A represents the extracellular pool of precursors of B and C, B represents the intracellular pool of precursors of C, and C represents the end products, in this case DNA, or RNA. The most favourable conditions for observing diffusion exchange of B between the inside and the outside of the cell are (1) a high steady-state concentration of B in the cell ($K_4 > K_2$); (2) a rapid exchange between inside and outside as compared with removal by synthesis ($K_3 > K_2$); and (3) a sufficient time of experiment. In the present case these conditions are best satisfied by observing slow growth over a long period as we have done.

We are therefore led to the conclusion, in the light of the results reported here, that RNA has a detectable rate of turnover in growing cells, and the possibility of turnover in DNA is not altogether excluded. Since the necessary information is not

available it is impossible to state whether this turnover involves all the RNA or DNA, or what the absolute rate may be in either case. The results do not exclude the possible existence of a transmissible block of DNA such as has been demonstrated to occur in bacteriophage by Levinthal (1956), but the existence of such a block remains to be proved and its size remains to be defined.

SUMMARY

1. The pattern of incorporation of [^{14}C]formate into the nucleic acids of strain L mouse fibroblasts was studied. The metabolic stability of the nucleic acids was also investigated by measuring the loss of ^{14}C from deoxyribonucleic acid, nuclear ribonucleic acid and cytoplasmic ribonucleic acid of labelled cells.

2. The cells are capable of synthesizing purines and thymine, but preferentially utilize adenosine, guanosine and thymidine when these are present in the medium. Adenosine is more readily incorporated than guanosine and conversion of adenine into guanine takes place more readily than does the reverse reaction.

3. Like some other tissues which have been studied *in vitro*, the cultured cells show a higher degree of incorporation of [^{14}C]formate into deoxyribonucleic acid thymine than into deoxyribonucleic acid purines when the medium contains plentiful amounts of purine and pyrimidine precursors. Incorporation into purines of both deoxyribonucleic acid and ribonucleic acid can be enhanced by addition to the medium of a mouse-liver extract or of 5-amino-4-imidazole carboxamide riboside. In the absence of preformed purines and pyrmidines in the medium twice as much [^{14}C]formate is incorporated into the deoxyribonucleic acid purines as into thymine.

4. When labelled cells are allowed to grow slowly in non-radioactive medium, activity is progressively lost from ribonucleic acid (in excess of the loss due to dilution). Cytoplasmic ribonucleic acid and nuclear ribonucleic acid behave similarly and some degree of turnover in both is suggested.

5. Little loss of ^{14}C from deoxyribonucleic acid is observed in similar conditions. However, when a large amount of thymidine is added to the medium the loss of activity from deoxyribonucleic acid thymine exceeds that found in control cultures, whereas the activities of adenine and guanine are unaffected. These observations suggest that there may be some turnover of deoxyribonucleic acid also but that this is obscured by reincorporation of breakdown products.

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Cellulolytic Enzymes from Sheep-Rumen Liquor Micro-organisms

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The enzymic conversion of cellulose into glucose is incompletely understood. The state in the rumen may be expected to be complex, since there are different organisms present and the enzyme may vary with the organism. Yet differences exist concerning even single and particular organisms: Grassmann, Zechmeister, Tóth & Stadler (1933) separated cellobiase as a distinct entity from extracts of *Aspergillus oryzae*, and indirect evidence for cellobiase as a separate enzyme in filtrates of *Myrothecium verrucaria* culture has been obtained by its selective inactivation on heating (Kooiman, Roelofsen & Sweeris, 1953; Aitken, Eddy, Ingram & Weurman, 1956). Whitaker (1953), however, obtained an enzyme preparation from *Myrothecium verrucaria* that was electrophoretically homogeneous and active towards both cellulose and cellobiose; he claims (Whitaker, 1956) that differential inactivation of cellobiase by heating represents a structural change in the enzyme which affects its activity in relation to the chain-length of the substrate. Evidence for both single- and multiple-enzyme components in extracts of *Aspergillus niger* has been found by chromatography (Stone, 1957), the fractions separated including one with activity towards two cellulose substrates as well as towards salicin, and others with activity towards either one cellulose substrate only or salicin only.

In the present investigation, the cellulolytic enzymes from sheep-rumen micro-organisms have been studied with cellobiose and carboxymethylcellulose as substrates. Substituted soluble derivatives of cellulose such as carboxymethylcellulose (Holden & Tracey, 1950), cellulose sulphate (Levinson, Mandels & Reese, 1951) and methyl cellulose (Aitken *et al.* 1956) have been extensively used as substrates for cellulase, but Siu (1951) and Halliwell (1957*a*, *b*) claim that preparations actively hydrolysing these substances do not necessarily attack native cellulose. In particular, the type of preparation used in the present investigation (obtained by butanol extracts of sheep-rumen micro-organisms) as well as an extract of the limpet *Patella vulgata* (found to be a good source of the enzyme) hydrolyse carboxymethylcellulose, but according to Halliwell (1957*a*, *b*) have negligible activity on filter paper and cotton fibres. To avoid confusion in this respect the enzyme attacking the soluble cellulose derivative will be termed carboxymethylcellulase rather than cellulase.

Conchie (1954) studied β-glucosidase from rumen liquor by using *o*-nitrophenyl β-glucoside as a substrate and showed gluconolactone to be a powerful inhibitor of the enzyme. The use of this inhibitor has been extended to the present study, a preliminary account of which has already been given (Festenstein, 1957).

EXPERIMENTAL

Materials. Sodium carboxymethylcellulose containing an average of 0·5 carboxymethyl/anhydroglucose (Cellofas B, Imperial Chemical Industries Ltd.) was kindly provided by Dr G. Halliwell. Cellobiose and glucono-1:4-lactone were obtained from L. Light and Co. Ltd. Both cellobiose and sodium carboxymethylcellulose were found to be chromatographically free from glucose and short-chain oligosaccharides.

Enzyme assay. The following procedure was usually followed: 0·2 ml. of each of 0·1 M-citric acid–0·2 M-Na_2HPO_4 buffer (pH 5·8; McIlvaine, 1921), substrate, inhibitor (where added) and enzyme were incubated at 37° in a total volume of 1 ml. (pH 6·1) in 15 ml. centrifuge tubes fitted with glass bulbs. After 1 hr. the reaction was stopped by adding the following reagents according to the particular method used for estimation of reducing sugar: 0·25 ml. of 0·5% $K_3Fe(CN)_6$ in 10% (w/v) Na_2CO_3 for the ferricyanide method; 0·27 ml. of 0·2 N-NaOH for the hypoiodite method; 1 ml. of 0·3 N-$Ba(OH)_2$ and 1 ml. of 5% (w/v) $ZnSO_4$ (standardized against each other as specified by Nelson, 1944) for the cuprimetric method. It was not found necessary to deproteinize the incubation mixtures for estimations by the ferricyanide and hypoiodite methods under the conditions used, provided that enzyme blanks were done. The cuprimetric method did not give consistent results for cellobiose in the presence of protein, so protein was removed as described.

Methods for estimating reducing sugar

Ferricyanide method. The method of Levvy (1946) was used.

Hypoiodite method. The method of Willstätter & Schudel (1918) was adapted as follows: after the addition of 0·27 ml. of 0·2 N-NaOH and 0·5 ml. of 0·04 N-iodine [freshly diluted from 0·1 N-iodine in 10% (w/v) KI], the tubes were corked and left at room temperature for 20 min., before acidification with 0·2 ml. of 4 N-H_2SO_4 and titration with 4 mN-$Na_2S_2O_3$ from a 10 ml. microburette. The tip of the burette was immersed in the solution, 3 drops of 1% starch solution

were added near the end point and air-stirring was used to complete the titration. Under these conditions up to 2 mg. of cellobiose reacted completely; usually 1 mg. was used as substrate. In experiments where 0·2 ml. of M-citric acid–2M-Na_2HPO_4 buffer, pH 5·5, was used in the initial incubation mixture (final pH 6·0), the reaction was terminated by the addition of 0·28 ml. of N-NaOH, and the procedure continued as before.

Cuprimetric method. The method of Nelson (1944), as modified by Somogyi (1952), was used as follows: after addition of 1 ml. each of $Ba(OH)_2$ and $ZnSO_4$ as described above (slightly more than sufficient to precipitate all the protein in 0·2 ml. of dialysed enzyme) and centrifuging at 1500 *g* for 5 min., 1 ml. portions (or fractions thereof) were taken for assay with 1 ml. of copper reagent.

Comparison of methods of estimating reducing sugars. Cellobiose gave about 53% of the reducing value of an equal weight of glucose for both the cuprimetric and hypoiodite methods; the ferricyanide method gave a much higher value (86%). Determination of reducing sugar after enzymic hydrolysis of carboxymethylcellulose expressed in terms of glucose gave similar values for the cuprimetric and hypoiodite methods; the ferricyanide method results were two to three times higher. A similar difference was observed by both Holden, Pirie & Tracey (1950) and Holden & Tracey (1950), when comparing a ferricyanide and a hypoiodite method. In the present investigation cellobiase estimations were therefore carried out by the hypoiodite and cuprimetric methods; the ferricyanide method was used only in the preliminary experiments involving carboxymethylcellulose.

Chromatography

Solutions obtained after enzymic hydrolysis of cellobiose and carboxymethylcellulose were analysed by descending-paper chromatograms on Whatman no. 1 paper. After incubation with enzyme, solutions were either placed in a boiling-water bath for 10 min. or, more generally, deproteinized with $Ba(OH)_2$ and $ZnSO_4$ as described above. In some cases the reaction was stopped by addition of 10% of the volume of 0·04M-$HgCl_2$ (Crook & Stone, 1957). Where concentrated cellobiose or glucose solutions (≥5%, w/v) had been incubated with enzyme, these were either applied to the paper without further treatment or after de-ionizing. All solutions to be concentrated were de-ionized by standing over a mixture of the resins Zeo-Karb 215 and Amberlite 1R-4B(OH) before being evaporated to dryness in a vacuum desiccator at room temperature. Generally the carboxymethylcellulose hydrolysates were redissolved in water so as to correspond to 0·5–1% solutions of glucose, as determined by estimation of reducing sugar. The following solvent systems were used: butanol–pyridine–water, (*a*) 6:4:3, by vol. (Chargaff, Levine & Green, 1948), (*b*) 6:2:3, by vol.; butanol–pyridine–water–benzene (5:3:3:1, by vol.) (De Whalley, Albon & Gross, 1951); acetic acid–propan-2-ol–water (10:58·5:31·5, by vol.) (Hash & King, 1954). Alkaline $AgNO_3$ reagent (Trevelyan, Procter & Harrison, 1950), aniline phosphate and benzidine in trichloroacetic acid (Bacon & Edelman, 1951) were used to detect the sugars on the chromatograms. The R_G value of a substance describes its rate of movement relative to D-glucose (R_G 1·0), determined on the same chromatogram.

Preparation of enzyme extract

The butanol-extraction method of Morton (1955), first used for rumen micro-organisms by Halliwell (1957*b*), was adapted as follows: a sample of rumen liquor (usually 1 l.), obtained from fistulated Cheviot sheep on a hay diet at 9·30 a.m., was strained through six layers of surgical gauze and centrifuged at 1500 *g* for 30 min. or 10 000 *g* for 20 min., depending on the micro-organisms required. After removal of the supernatant layer the precipitate was suspended in 1% NaCl solution, the final volume being chosen to give a concentrated suspension that was not too viscous (5–10% of the original volume of rumen liquor). Butanol was added slowly, with stirring, to 10% (by vol.) over 10 min. and then to 30% (by vol.) over the next 5 min. The resulting mixture was centrifuged at 10 000 *g* for 20 min. and the aqueous layer (3–5% of the original volume of rumen liquor) re-centrifuged (after precooling to 1°) until clear.

The enzyme extracts stored at 1° retained their carboxymethylcellulase activity for at least several months. Butanol was usually removed by dialysis in sacs of Visking cellulose tubing against distilled water at 1°, for 18–24 hr. before use.

The organisms centrifuged at 1500 *g* [described microscopically by Karunairatnam & Levvy (1951)] were found to contain about 85% of the total extractable carboxymethylcellulase, so extracts of this particular fraction were generally used, except for determination of the pH optimum of the enzyme (see below) where a sample of total organisms was extracted. Conchie (1954) found a similar distribution of *o*-nitrophenyl β-glucosidase activity, with fractions of intact organisms.

The mean protein content of six enzyme preparations from three different sheep, dialysed extracts obtained from organisms centrifuged at 1500 *g*, was 2·6 mg./ml. (range 0·8–3·5), as determined by the method of Lowry, Rosebrough, Farr & Randall (1951), standardized against crystalline bovine-plasma albumin. For one particular sample of rumen liquor the protein content of the dialysed extract from the smaller organisms (centrifuged between 1500 and 10 000 *g*) was one-third of that of the dialysed extract from the organisms centrifuged at 1500 *g*.

RESULTS

Effect of pH *on enzyme activity.* The variation of carboxymethylcellulase activity with pH is shown in Fig. 1. The multi-peaked curve resembles that obtained by Conchie (1954) for *o*-nitrophenyl β-glucosidase, with intact mixed rumen micro-organisms. A limpet-enzyme preparation provided by Dr J. Conchie (cf. Conchie, Levvy & Marsh, 1956; Conchie & Levvy, 1957), which was found to possess carboxymethylcellulase activity, showed a similar pH–activity curve (Fig. 1). The optimum pH determined for cellobiase (0·1% substrate concentration) was found to lie between pH 5 and 6

*Differentiation of cellobiase and carboxymethylcellulase by glucono-*1:4*-lactone*

Inhibition of cellobiase by gluconolactone. By the use of short incubation periods (30–60 min.) and concentrations of substrate up to 0·4%, 2·5 mM-

lactone was found sufficient to produce complete inhibition of cellobiase; for concentrations up to 0·2 %, 0·5 mM-lactone was mostly sufficient. For 5 % cellobiose solutions, 2·5 mM-lactone produced nearly complete inhibition of enzymic hydrolysis, as determined chromatographically (see later).

The effect of time on inhibition by lactone, studied in previous experiments, is shown in Fig. 2. It can be seen that inhibition was not sustained for periods longer than 60 min. and investigations have to be carefully controlled on this account.

An evaluation of K_i for gluconolactone was not possible because of non-linear hydrolysis in the presence of lactone, similar to the lag phase sometimes observed for hydrolysis of cellobiose by diluted enzyme (see Fig. 2).

Inhibition of carboxymethylcellulase by gluconolactone. At a substrate concentration of 0·4 %, inhibition was detected with 0·02 mM-lactone and rose rapidly with lactone concentration to 60 %, where it remained constant for lactone concentrations $\geqslant$ 2·5 mM (Fig. 3). When 2·5 mM-lactone was used for a range of substrate concentrations from 0·4 to 0·04 %, a value of about 50 % inhibition was obtained for practically the whole range.

Effect of preheating of enzyme on inhibition of carboxymethylcellulase by gluconolactone. When an

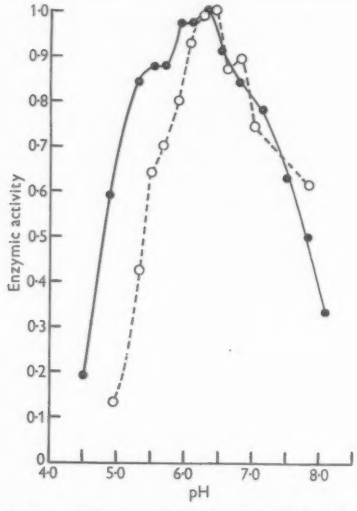

Fig. 1. Effect of pH on carboxymethylcellulase activity. ●, Results for an enzyme extract obtained from a sample of total rumen micro-organisms, washed with NaCl. Reaction mixtures (1 ml.) contained 0·2 % of carboxymethylcellulose, 1:100 enzyme (corresponding to about fivefold dilution of rumen liquor) and 0·2 ml. of 0·1 M-citric acid–0·2 M-Na_2HPO_4 buffer of appropriate pH. An activity of 1·0 corresponds to liberation of reducing substances equivalent to 0·06 ml. of 11 mN-$Ce(SO_4)_2$ (or 24 μg. of glucose). ○, Results for a limpet extract (30 g. of wet tissue/100 ml.) diluted 1:2000 in reaction mixtures as above; in this case an activity of 1·0 corresponds to a titre of 0·095 ml. of 11 mN-$Ce(SO_4)_2$.

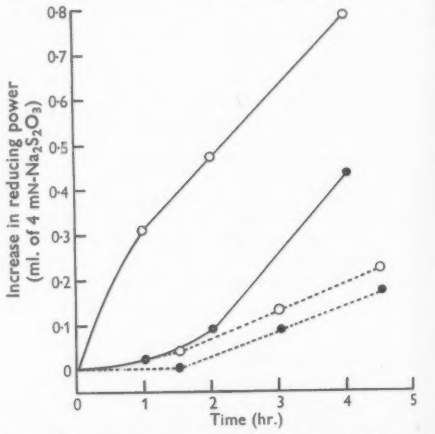

Fig. 2. Progress of enzymic hydrolysis of cellobiose and the effect of gluconolactone. ○—○ and ●—● represent the hydrolysis of 0·1 % cellobiose in the absence and the presence of 10 mM-gluconolactone respectively; ○- - -○ and ●- - -● represent the hydrolysis of 0·05 % cellobiose by diluted enzyme (1:5) in the absence and the presence of 0·5 mM-gluconolactone respectively. Incubation was carried out in 0·2 M-citric acid–0·4 M-Na_2HPO_4 buffer, pH 6·0. An increase in reducing power of 1·40 ml. of 4 mN-$Na_2S_2O_3$ corresponds to the liberation of 1 mg. of glucose.

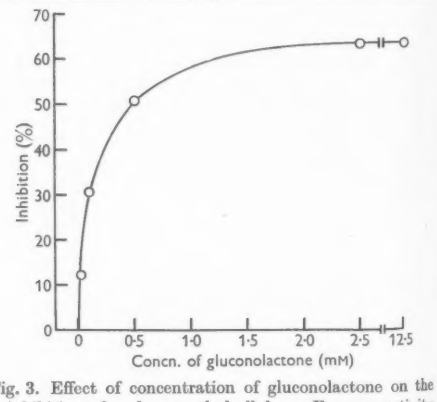

Fig. 3. Effect of concentration of gluconolactone on the inhibition of carboxymethylcellulase. Enzyme activity was determined by measurement of the reducing substances liberated from 0·4 % sodium carboxymethylcellulose after incubation for 1 hr. (as determined by the cuprimetric method).

enzyme preparation was kept at 65° for 10 min., cellobiase activity was lost, carboxymethylcellulase activity was reduced to 15 % of its initial value and inhibition of carboxymethylcellulase by 2·5 mM-gluconolactone was completely abolished.

Chromatographic analysis of cellobiose and carboxymethylcellulose hydrolysates, after enzyme action in the presence and the absence of gluconolactone. Chromatograms of hydrolysates of 0·2 % cellobiose (made after about 20 % hydrolysis of substrate, and usually concentrated 10–25 times) revealed only glucose and cellobiose when examined with aniline phosphate; silver nitrate reagent showed a trace of a substance, R_G 0·22 (solvent, butanol–pyridine–water–benzene), which was probably formed by a transferase reaction. Transferase activity could be readily detected by incubating enzyme with 5 % (w/v) solutions of cellobiose (cf. Crook & Stone, 1957); three other substances besides glucose were produced, which can be tentatively identified as 6^2-β-glucosylcellobiose, gentiotriose and gentiobiose (arising in that order), on the basis of the R_G values in different solvent systems compared with the data of Crook & Stone (1957). Gluconolactone (2·5 mM) inhibited transferase activity as well as greatly diminishing production of glucose.

Chromatograms of carboxymethylcellulose hydrolysates sprayed with aniline phosphate or benzidine in trichloroacetic acid revealed glucose as the predominant product, along with traces of cellobiose. Occasionally traces of a substance producing a pink colour with aniline phosphate (R_G 1·4, butanol–pyridine–water–benzene) were found; possibly this was xylose. The intensity of the glucose spot on the chromatogram generally corresponded to less than would be expected if the total reducing material was glucose. If 2·5 mM-gluconolactone was present during the incubation of carboxymethylcellulose with the enzyme, the amount of glucose, although very much reduced, was still detectable; cellobiose sometimes showed a slight increase but no other substances were found.

The more sensitive silver nitrate reagent detected several other substances on the chromatograms, including gluconolactone; up to three different spots with R_G values varying from 0 to 0·11 (butanol–pyridine–water–benzene) were found but when gluconolactone (R_G 0·14) was present it overlapped with and possibly obscured intensity changes in these spots. Gluconolactone was only partially removed by Amerlite 1R-4B(OH) resin added after deproteinization with barium hydroxide–zinc sulphate, even when this resin was the first added and the pH rose to 8·6. In acetic acid–propan-2-ol–water as developing solvent gluconolactone produced a spot which almost coincided with glucose, but did not interfere with any of the others [it was found advisable to deproteinize with barium hydroxide–zinc sulphate and then to deionize to ensure that the lactone did not interfere]; the results obtained with the acetic acid-containing solvent are summarized in Table 1.

DISCUSSION

The presence of a multi-peaked pH–activity curve for carboxymethylcellulase suggests either differences in respect of the same enzyme for different organisms or alternatively more than one type of enzyme. Jermyn (1952*a*) found more than one peak in the pH–activity curves for *Aspergillus oryzae* enzyme acting on cellobiose, salicin and carboxymethylcellulose; he subsequently showed (Jermyn, 1952*b*) by chromatographic and electrophoretic analysis that there were at least eight components, with varying degrees of specificity towards different β-glucosidic substrates. In contrast with this finding a *Stachybotrys atra* preparation, which showed a multipeaked pH–activity curve for *p*-nitrophenyl β-glucoside as substrate, yielded only one component on chromatography and electrophoresis (Jermyn, 1955*a*, *b*).

The inhibition of carboxymethylcellulase by gluconolactone does not exceed 60 %, as determined by estimation of reducing sugar. The maximum value is attained by 2·5 mM-lactone (see

Table 1. *Chromatographic analysis of* 0·4–0·8 % *carboxymethylcellulose hydrolysates with the solvent system acetic acid–propan-2-ol–water*

The results represent findings for five different enzyme preparations, all incubated for 1 hr. Reducing substances were detected by $AgNO_3$; + represents a barely detectable amount, + + + a definite spot of weak intensity. Neither glucose nor spots stretching from R_G 0·0 to 0·2, which showed no special character, are included here.

Substances detected (R_G values)	Incubation without lactone	Incubation with lactone (2·5 mM)	Notes
0·23	–	+ +	Found consistently
0·58	+ +	+ +	Did not always occur
0·71	+ +	+	Did not always occur
0·80	+ + +	+ + + (+)	Cellobiose

Fig. 3), which is sufficient to produce complete inhibition of cellobiase for concentrations of cellobiose up to 5%. This suggests that two types of enzyme are involved in the production of reducing sugar from carboxymethylcellulose, one of which is inhibited by lactone in a similar manner to cellobiase. In accord with this interpretation, Reese & Mandels (1957) found that the carboxymethylcellulase activity of culture filtrates of *Myrothecium verrucaria*, *Pestalotiopsis westerdijkii* and *Trichoderma viride* was unaffected by gluconolactone when measured under conditions where the effects due to cellobiase were eliminated.

The results obtained by treating chromatograms of carboxymethylcellulose hydrolysates with aniline phosphate or benzidine spray are in agreement with the findings of Kitts & Underkofler (1954), who chromatographed carboxymethylcellulose hydrolysates obtained with rumen micro-organisms and cell-free preparations and found glucose to be the chief product, with traces of cellobiose and xylose. Since gluconolactone inhibits cellobiase, it might be expected that the products obtained from extracts of rumen micro-organisms incubated with carboxymethylcellulose in the presence of lactone would be intermediates formed in the normal process of hydrolysis. It was found (Table 1) that cellobiose increased only slightly and not invariably, and small amounts of a substance of R_g 0·23 (in the acetic acid-containing solvent) were produced, as detected by the silver nitrate reagent. From the data of Hash & King (1954) cellotetraose has R_g 0·35 in the same solvent system, and interpolated values for cellopentaose and cellohexaose are R_g 0·22 and 0·14 respectively. On this basis it would appear that the additional substance appearing in the presence of lactone may be cellopentaose. The substances of R_g 0·58 and 0·71 (Table 1) did not always occur; the former may be cellotriose (R_g 0·53) and the latter a product of transferase activity, inhibited by gluconolactone.

Kooiman *et al.* (1953) found that a soluble cellulose dextrin hydrolysed by *Myrothecium verrucaria* enzyme produced a range of sugars from glucose to cellotetraose, with traces of cellopentaose and cellohexaose, as identified by paper chromatography. When the enzyme was preheated at 100° for 15 min., it still produced the sugars, but cellobiose was increased in amount, and so apparently was cellopentaose which, previously recorded as doubtful, now appears as definitely present. These findings accord with the results of the present investigation.

Levinson *et al.* (1951) detected cellobiose as the initial product of hydrolysis of cellulose sulphate by *Myrothecium verrucaria* and *Trichoderma viride* culture filtrates which were low in 'β-glucosidase'. Aitken *et al.* (1956) similarly established that heating of *Myrothecium verrucaria* enzyme (to abolish cellobiase activity) diminished production of glucose from methyl cellulose, but the absolute amount of 'cellobiose etc.' as determined by estimation of reducing sugar after yeast fermentation, was not substantially increased. It would appear that this heating of enzyme produced a similar result to inhibition by lactone in the present study.

The conclusion to be drawn here is that production of glucose from carboxymethylcellulose practically ceases if a cellobiase-type of enzyme is inhibited; cellobiose does not increase markedly under these conditions, suggesting either that glucose is split off from carboxymethylcellulose directly, and that this mechanism is inhibited by lactone, or else that inhibition of hydrolysis of cellopentaose and possibly higher homologues occurs and that this hydrolysis gives rise to glucose as well as cellobiose as described by Whitaker (1956). The rate of hydrolysis of cellobiose by *Myrothecium verrucaria* enzyme was shown by Whitaker (1956) to be extremely slow, and that of cellotriose to be moderately fast, in comparison with the extremely fast rate of hydrolysis of cellopentaose and cellohexaose. Grassmann *et al.* (1933) showed that, for *Aspergillus oryzae*, 'polysaccharase' (as distinct from 'oligosaccharase' or cellobiase) hydrolysed cellohexaose to a certain extent but had hardly any effect on cellotetraose and lower oligoglucosides.

SUMMARY

1. Cell-free extracts of sheep-rumen micro-organisms have been used to study the hydrolysis of cellobiose and sodium carboxymethylcellulose, by determination of the reducing sugar liberated.

2. Controlled inhibition studies with gluconolactone have shown cellobiase to be completely inhibited by 2·5 mM-lactone, for concentrations of cellobiose up to 5% (w/v). Inhibition of hydrolysis of sodium carboxymethylcellulose reaches a constant value of 60% at a lactone concentration where cellobiase is completely inhibited.

3. Chromatographic analysis of cellobiose hydrolysates has shown that transferase activity is barely detectable in 0·2% cellobiose solutions, but becomes appreciable in 5% cellobiose, and three oligosaccharides have then been detected. Transferase activity is inhibited by gluconolactone.

4. Chromatographic analysis of sodium carboxymethylcellulose hydrolysates has shown glucose to be the predominant product, along with traces of cellobiose, and possibly xylose. In the presence of gluconolactone, production of glucose is almost completely inhibited and the amounts of cellobiose are sometimes slightly increased; a small amount of a substance which may be cellopentaose has been detected with a silver nitrate reagent.

Thanks are due to Dr G. A. Levvy for his stimulating and critical interest in this work; to Dr G. Halliwell for suggesting the butanol method of enzyme extraction, assistance at the commencement of the investigation and for providing the samples of rumen liquor used; to Dr B. H. Howard for advice on chromatography; to Mr I. Strachan and Miss E. F. Wallace for technical assistance. Acknowledgement is also due to several colleagues for comments on the manuscript.

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Studies on Sulphatases

21. THE ANOMALOUS KINETICS OF ARYLSULPHATASE *A* OF HUMAN TISSUES: THE ANOMALIES*

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The anomalous kinetics shown by partially purified preparations of the arylsulphatase *A* of ox liver when acting on dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS) were first noted by Roy (1953*a*, *b*). When measured over an incubation period of 1 hr. the velocity of the enzymic reaction was linearly related to enzyme concentration only when the latter was raised to the power of 3/2. This effect was attributed to the ability of the enzyme to exist in several polymeric forms. Similar anomalous kinetics were observed by Dodgson, Spencer & Wynn (1956) for the corresponding enzyme of human liver, and it was subsequently possible to show (Dodgson & Spencer, 1956*a*) that the fundamental anomaly was that the enzymic reaction was not of zero order when low concentrations of enzyme were used. These findings were later confirmed for the ox enzyme (Roy, 1956). Meanwhile, it was noted (Roy, 1956; Roy & Kerr, 1956) that the NCS used in these studies contained appreciable amounts of a sulphated impurity which was identified as nitropyrogallol disulphate. When NCS which had been 'purified' via the methylene-blue salt or by paper electrophoresis was used as assay substrate, the ox-liver enzyme no longer exhibited anomalous kinetics.

* Part 20: Spencer (1958).

Subsequently, Dodgson & Spencer (1956*b*) showed that the introduction of contaminating materials during these purification procedures was the fundamental reason for the disappearance of the anomalous kinetics. These workers eliminated nitropyrogallol disulphate from preparations of NCS by repeated recrystallization of the latter in the form of the monopotassium salt. Human arylsulphatase *A* still exhibited anomalous kinetics towards the purified substrate.

The properties of the human enzyme have been extensively investigated in these laboratories but have not previously been reported in detail because, until recently, it was difficult to provide an adequate explanation of the findings. However, Roy (1957) has recently summarized his findings for ox-liver arylsulphatase *A*, and it now seems important to present this account of the properties of the human enzyme in view of the many additional observations made.

MATERIALS AND METHODS

Substrates. Monopotassium *p*-nitrophenyl sulphate (NPS) and monopotassium *p*-acetylphenyl sulphate (APS) were prepared by the method of Burkhardt & Lapworth (1926). Dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) was prepared according to the directions of Roy (1953*a*) and purified by the method of Dodgson & Spencer (1956*b*). All substrates were repeatedly recrystallized until free from inorganic sulphate.

Preparations of arylsulphatase A

Concentrates of human arylsulphatase *A* were made in several ways, but, irrespective of the method of preparation, similar results were always obtained.

Preparation 1. Human liver, obtained within 48 hr. after death, was acetone-dried (see Dodgson, Rose, Spencer & Thomas, 1957), and the resultant powder sieved (40 mesh) to remove connective tissue. The powder (60 g.) was suspended in 300 ml. of 0·1 M sodium acetate–acetic acid mixture, pH 7, with the aid of a Townson and Mercer macerator and the suspension was incubated at 37·5° for 15 min. before centrifuging for 15 min. at 2000 *g*. The debris was washed with a further 200 ml. of the acetate mixture and, after centrifuging as before, the two supernatants were combined and further clarified by centrifuging at 5000 *g* for 20 min. The clear supernatant was cooled to 0° and acetone, previously cooled to −5°, was added with stirring over a period of 30 min. until the final concentration of acetone was 45% (v/v). After standing for 30 min. at −5° the precipitate was removed by centrifuging at 2000 *g* and −5°. To the clear supernatant was added 4 ml. of 0·2 M-$CaCl_2$ soln. and acetone was added as before until the concentration was 50% (v/v). After standing at −5° for 30 min. the precipitate was separated by centrifuging and discarded. The concentration of acetone in the clear supernatant was then increased to 60% (v/v) and, after standing as before, the precipitated material was separated by centrifuging, dissolved in 30 ml. of water and dialysed overnight against tap water. The diffusate was clarified by centrifuging, the pH adjusted to 5 with a trace of acetic acid, and solid ammonium sulphate was slowly added (with stirring) until the solution was 35% saturated with regard to ammonium sulphate. The pH was readjusted to 5 and the mixture allowed to stand for 5 hr. at room temperature. The precipitate was separated by centrifuging, dissolved in 20 ml. of water, dialysed for 18 hr. against tap water and subsequently for 24 hr. against several changes of distilled water (8 × 500 ml. at 2°). The cloudy solution was clarified by centrifuging and the colourless clear supernatant stored in the frozen state. The protein content of the preparation was usually in the region of 0·5 mg./ml. when measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Occasionally preparation 1 was further dialysed against several more changes of distilled water at 2°. Under these circumstances a precipitate sometimes appeared which, when extracted with 0·5 M sodium acetate–acetic acid buffer, pH 5, gave a solution which contained the bulk of the enzyme activity (preparation 1*A*). This preparation exhibited the same anomalous kinetics as preparation 1 and had about ten times the activity/wt. of protein. However, the preparation was somewhat unstable.

Preparation 2. This was identical with the first preparation as far as the precipitation of the 50–60% (v/v) acetone fraction. This fraction was dissolved in water (20 ml.), dialysed overnight against tap water, clarified by centrifuging and concentrated to low bulk by freeze-drying. The concentrated solution was then subjected to horizontal-paper electrophoresis on several strips of Whatman no. 100 filter paper which had previously been treated with cetylpyridinium bromide as described by Dodgson & Spencer (1956*c*). Electrophoresis was carried out in the refrigerator for 18 hr. at 210 v in 0·1 M sodium acetate–acetic acid buffer, pH 5. Subsequently, arylsulphatase *A* was located on one of the strips (see Dodgson, Spencer & Thomas, 1955) and the enzyme was then eluted from the appropriate portion of the remaining strips with water. After concentrating to a suitable volume by freeze-drying, the enzyme solution was dialysed against tap water followed by distilled water as described for preparation 1.

Preparation 3. The arylsulphatase *A* present in freshly voided urine was separated from arylsulphatase *B* by the electrophoretic procedure of Dodgson & Spencer (1956*c*) and was subsequently eluted from several strips of filter paper with water. The eluates were combined and concentrated to low bulk by freeze-drying before dialysing as described for the other preparations.

Paper electrophoresis of the three preparations showed that in no case was arylsulphatase *B* (see Dodgson *et al.* 1956) present.

Other arylsulphatase preparations. The arylsulphatase of *Alcaligenes metalcaligenes* was concentrated according to the method of Dodgson, Spencer & Williams (1955), limpet (*Patella vulgata*) arylsulphatase was concentrated to give preparation *D* (see Dodgson & Spencer, 1953) and Taka-diastase (Parke, Davis and Co. Ltd.) was used as a source of fungal arylsulphatase. Human arylsulphatase *B* was prepared by the method of Dodgson & Wynn (1958), and the digestive juice of *Helix pomatia* was used as the source of snail arylsulphatase. All the preparations were dialysed for 18 hr. against tap water and subsequently for 24 hr. against several changes of distilled water (8 × 500 ml. at 2°).

Enzyme assay. The following method was employed in all cases irrespective of which substrate or enzyme preparation was used. The enzyme solution (3 ml., contained in a stoppered vessel) was pre-incubated for 3 min. at 37·5° before adding, as quickly as possible, an equal volume of a solution of the appropriate substrate (in 0·5 M sodium acetate–acetic acid buffer, adjusted to the appropriate pH) which had previously been warmed to 37·5°. Samples (0·4 ml.) were withdrawn at suitable time intervals and added immediately to 2 ml. of N-NaOH. After mixing, the liberated phenols were measured spectrophotometrically with the appropriate wavelength of maximum absorption (see Dodgson & Spencer, 1957*a*). Suitable control determinations were made.

Mention has already been made that similar results were obtained with all three enzyme preparations. However, for obvious reasons, preparation 1 was generally preferred and was used in all the experiments described below. When NCS was the substrate the kinetic anomalies were shown to best effect when enzyme activity was such that 3–9 μg. of 4-nitrocatechol was liberated in the initial 10 min. period/0·4 ml. of enzyme–substrate mixture, and the concentration of enzyme necessary to achieve this was determined by preliminary experiment. Generally speaking, the enzyme preparation had to be diluted about 10 times, although the activity varied appreciably from liver to liver.

RESULTS

Influence of various factors on the enzyme reaction

Time and enzyme concentration. Fig. 1 shows the time–activity curves for different concentrations of arylsulphatase *A* acting on 5 mM-NCS in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5. At the lowest enzyme concentration there is an initial rapid enzyme reaction (stage I) which is followed by a period of very low but steady enzyme activity (stage II). At the higher enzyme concentractions, however, this second phase is not maintained and enzyme activity slowly increases again until it again reaches a steady rate (stage III), which, however, is less than that shown in stage I. At higher concentrations of enzyme than those shown in Fig. 1, the curve tends towards (but never achieves) a straight line. Similar curves are obtained after pre-incubation of the enzyme solution for 1 hr. at 37·5° before adding substrate, thus indicating that the phenomenon is at least partly dependent on the presence of substrate.

Fig. 2 shows the enzyme concentration–activity curves plotted from the data shown in Fig. 1. These curves vary appreciably with the time of incubation but in no case are they linear. Other experiments showed the relationship between enzyme concentrations and activity to be approximately linear only during the initial 2 or 3 min. of the reaction. The problems associated with the quantitative assay of arylsulphatase *A* in its purified form will be obvious from a consideration of Figs. 1 and 2.

Substrate concentration. Increasing substrate concentration results in increased enzyme activity during the initial stage (stage I) of the incubation

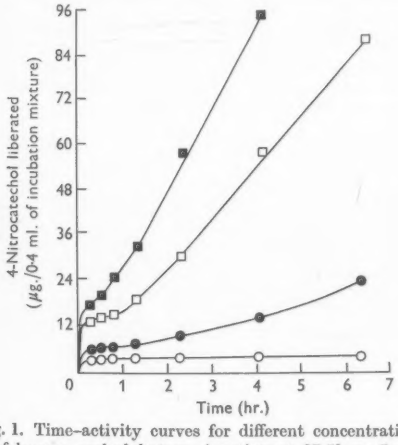

Fig. 1. Time–activity curves for different concentrations of human arylsulphatase *A* acting at 37·5° on 5 mM-NCS in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5. The lowest relative enzyme concentration (RE) used was obtained by diluting preparation 1 fifteen times. ○, RE = 1; ●, RE = 2; □, RE = 4; ■, RE = 6.

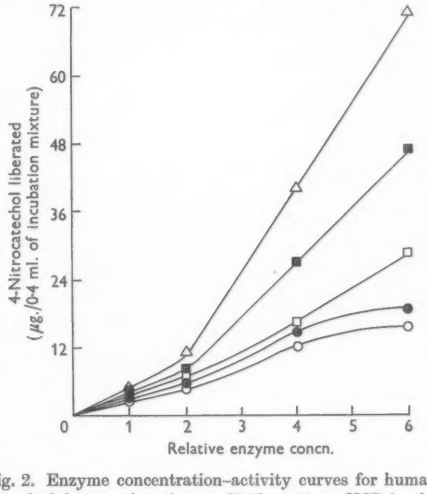

Fig. 2. Enzyme concentration–activity curves for human arylsulphatase *A* acting at 37·5° on 5 mM-NCS in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5, for various periods of time. The curves are plotted from the data shown graphically in Fig. 1. ○, 15 min.; ●, 45 min.; □, 60 min.; ■, 120 min.; △, 180 min.

period. In contrast, at substrate concentrations greater than 4 mM, increasing substrate concentration decreased the rate of enzymic hydrolysis during the later stages. Fig. 3 shows the substrate concentration–activity curve plotted for an incubation period of 10 min. (i.e. during stage I), from which it can be seen that the enzyme appears to be behaving according to the normal Michaelis–Menten equation. Fig. 3 also shows the effect of substrate concentration on the activity of the enzyme during stage III. Actually, the amounts of 4-nitrocatechol liberated between 90 and 130 min. were taken as a measure of the activity during this stage. The curve obtained is typical of that for inhibition by excess of substrate.

pH. The time course of the enzyme reaction is markedly affected by variation of pH, and hence pH–activity curves for arylsulphatase *A* vary considerably with the length of incubation period used. Fig. 4 shows the pH–activity curves plotted for various time intervals. During stage I the curves show two peaks of activity at pH 4·4 and 5·0 respectively but during stages II and III these peaks merge into a single peak at pH 4·4 which, in turn, shifts towards the region of pH 4·7 as the incubation proceeds. When the concentration of enzyme was increased so that approx. 15 μg. of 4-nitrocatechol was liberated in the initial 10 min. period/0·4 ml. of enzyme–substrate mixture, a single pH optimum was obtained in the region of pH 4·8 (Fig. 4).

Temperature. The time–activity curves obtained at various temperatures for two different concentrations of enzyme acting on 5 mM-NCS at pH 5 are shown in Fig. 5. During the first few minutes of the reaction the enzyme behaves in the expected manner towards increase in temperature. However, as the reaction proceeds, the time–activity curves obtained at 20·5° and 30·5° cross those obtained at 37·5° and 50·5°, although the point at which this occurs varies somewhat with the enzyme concentration used in the experiment. Although not shown in Fig. 5, the greater rate of activity at 20·5° is prolonged for several hours, but during this

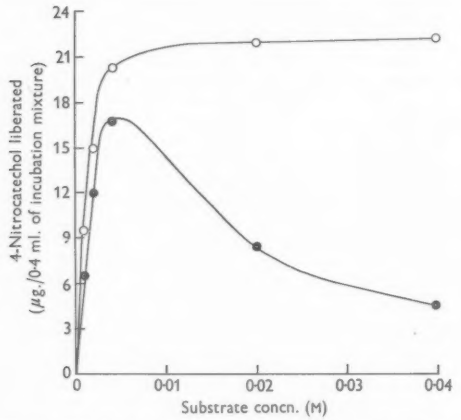

Fig. 3. Effect of substrate (NCS) concentration on the activity of human arylsulphatase *A* during stages I and III of the enzymic reaction. Enzyme activity was determined in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5, and the enzyme solution used was preparation I diluted five times. ○, Stage I (an incubation period of 10 min.); ●, stage III (plotted from the amounts of 4-nitrocatechol liberated between 90 and 130 min. of the incubation period; see text).

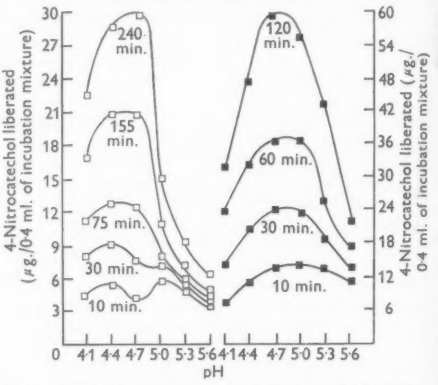

Fig. 4. pH–Activity curves for human arylsulphatase *A* plotted for various time intervals. The enzyme was acting on 5 mM-NCS in 0·25 M sodium acetate–acetic acid buffer at 37·5°. □, Preparation 1 diluted ten times; ■, preparation 1 diluted five times.

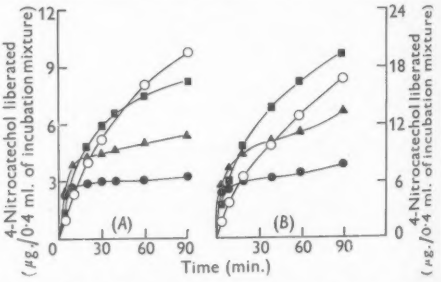

Fig. 5. Effect of temperature on the time–activity curves for human arylsulphatase *A* acting on 5 mM-NCS in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5. The enzyme solutions used were (*A*) preparation 1 diluted fourteen times and (*B*) preparation 1 diluted seven times. ○, 20·5°; ■, 30·5°; ▲, 37·5°; ●, 50·5°.

time the 37·5° time–activity curve shows an increasing upward trend and approaches the 20·5° curve and finally crosses it. This clearly indicates that thermal destruction of the enzyme is not an important factor in the production of these anomalous curves.

Behaviour of the enzyme towards other arylsulphatase substrates

Arylsulphatase *A* is a type II arylsulphatase (see Dodgson & Spencer, 1957*b*) which exhibits considerable affinity and activity towards NCS but only relatively low affinity and activity towards simpler arylsulphates such as NPS and APS. Nevertheless, by suitably adjusting the experimental conditions it can be shown that the enzyme also exhibits anomalous kinetics towards these other substrates. The pH–activity curves obtained for various incubation periods when the enzyme is acting against 50 mM-NPS are given in Fig. 6. Again two peaks of activity (at 5·3 and 6·1) are obtained over short incubation periods and these peaks give rise to a single peak in the region of pH 5·3 when the incubation period is prolonged. Similar results are obtained when APS is used as the substrate. Fig. 7 shows the time–activity curves obtained for different concentrations of enzyme acting on 50 mM-NPS at pH 6·1 and the enzyme concentration–activity curves for various incubation periods. The curves are very similar to those obtained with NCS, although it will be noted that the conditions of pH and enzyme and substrate concentration which have been employed to obtain these curves are very different from those necessary when NCS was the substrate. Similar results can be obtained with APS.

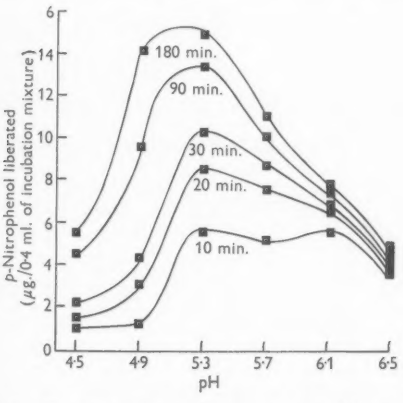

Fig. 6. pH–Activity curves for human arylsulphatase *A* for various time intervals when acting at 37·5° on 50 mM-NPS in the presence of 0·25 M sodium acetate–acetic acid buffer. The enzyme solution used was preparation 1 diluted three times.

Behaviour of other arylsulphatases towards NCS

Ox (Roy, 1953*a*) and rat (Dodgson & Spencer, unpublished work) tissues are known to possess an enzyme corresponding to human arylsulphatase *A*, and it seems likely that similar considerations will apply to other mammalian species. Arylsulphatases from other sources do not exhibit anomalous

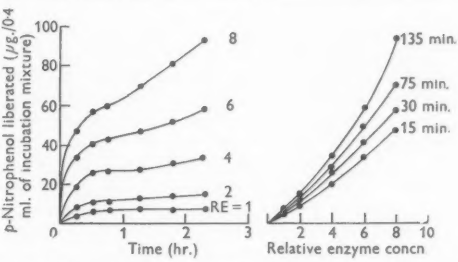

Fig. 7. Time–activity curves for different enzyme concentrations and enzyme concentration–activity curves for different time intervals for human arylsulphatase *A* acting on 50 mM-NPS in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 6·1. Incubation was at 37·5° and the lowest relative concentration of enzyme (RE) used was concentrated (×2, by freeze-drying) preparation 1 diluted eight times.

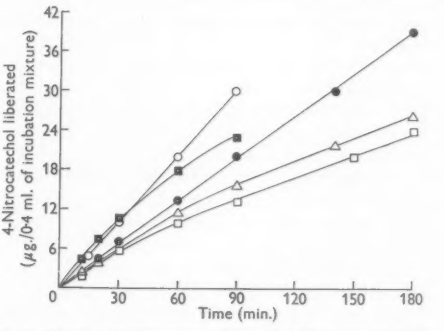

Fig. 8. Time–activity curves for arylsulphatases of different origin acting on NCS at 37·5° in the presence of 0·25 M sodium acetate–acetic acid buffer. The concentrations of the enzymes used were those necessary to liberate 3–9 μg. of 4-nitrocatechol/0·4 ml. of incubation mixture/20 min. as determined by preliminary experiments. The final concentrations of NCS and the pH at which the experiments were carried out are given below in parentheses. ○, Taka-diastase (2·5 mM, pH 6·0); ●, *Alcaligenes metalcaligenes* (15 mM, pH 8·0); □, *Patella vulgata* (10 mM, pH 5·6); ■, *Helix pomatia* (15 mM, pH 7·5); △, human arylsulphatase *B* (20 mM, pH 6·0).

kinetics towards NCS. Fig. 8 shows the time–activity curves obtained for the arylsulphatases of Taka-diastase (*Aspergillus oryzae*), *Alcaligenes metalcaligenes*, *Patella vulgata* and *Helix pomatia*, with the appropriate conditions of pH and substrate concentration. Also included in the figure is human arylsulphatase *B*. In no case were anomalous time–activity curves obtained. Similar results were obtained when the experiments were carried out at pH values which varied on either side of the appropriate optimum by up to 2 pH units.

DISCUSSION

Mention has already been made in the introduction to this paper that the anomalous kinetics of human (and ox) arylsulphatase *A* cannot be due to either polymerization of enzyme or the presence of impurities in the substrate as was originally suggested by Roy (1953*b*, 1956). The observation (Roy, 1953*b*) that the activity of the ox enzyme was linearly related to enzyme concentration raised to the power 3/2 resulted from the fortuitous selection of an incubation period of 1 hr. and is in no way a measure of enzyme polymerization, and the fact that the enzyme gives anomalous kinetics with purified NCS as well as other arylsulphates disposes of the second theory. Roy (1957) failed to obtain the anomalies characteristic of the hydrolysis of NCS when NPS was used as the substrate but this can almost certainly be attributed to the fact that by studying the reaction at pH 5 this author was inadvertently selecting a pH unfavourable to the anomalous kinetics, and indeed curves similar to those observed by Roy (1957) can be obtained with the human enzyme if the enzyme experiments are carried out at pH 5·0 rather than at 6·1. The finding that arylsulphatases from other sources do not exhibit anomalous kinetics towards NCS also suggests that substrate impurities cannot be responsible for the anomalies exhibited by arylsulphatase *A*.

The weight of the evidence clearly indicates that the anomalous kinetics are a feature of all mammalian arylsulphatases *A*. It is possible, of course, that the presence of two arylsulphatases in the enzyme preparations could give rise to anomalous kinetics of this sort but there are reasons for discarding this possibility and these will be mentioned in the next paper (Baum & Dodgson, 1958). Assuming then that the anomalies are peculiar to the enzyme it seems clear that, under the influence of substrate, a relatively slow inactivation of the enzyme occurs and this is in turn followed by an apparent partial reactivation of the enzyme. The succeeding communication provides further examples of the way in which these processes may be modified and suggests a possible explanation of the phenomenon.

SUMMARY

1. Preparations of human arylsulphatase *A* exhibit anomalous time–activity curves when incubated with dipotassium 2-hydroxy-5-nitrophenyl sulphate and other aryl sulphates.

2. These anomalies can be considerably modified by varying the pH or temperature of the enzyme reaction and by varying the concentration of enzyme and substrate.

3. The anomalous kinetics appear to be a feature peculiar to mammalian arylsulphatases *A* and are not shown by human arylsulphatase *B* or the arylsulphatases of limpets, *Alcaligenes metalcaligenes*, Taka-diastase and *Helix pomatia*.

This work was aided by the Medical Research Council, the Royal Society, the Wellcome Trust and Imperial Chemical Industries Ltd. One of us (H.B.) is grateful to the M.R.C. for a research assistantship. We are indebted to Dr R. Henry for a gift of the digestive juice of *Helix pomatia* and to Professor J. Gough of the Department of Pathology of the Welsh National School of Medicine for specimens of human liver.

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Studies on Sulphatases

22. THE ANOMALOUS KINETICS OF ARYLSULPHATASE *A* OF HUMAN TISSUES: INTERPRETATION OF THE ANOMALIES*

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The anomalous kinetics of human arylsulphatase *A* have been described in the preceding paper (Baum, Dodgson & Spencer, 1958). This paper is concerned with the effect of various other factors on the activity of the enzyme and suggests a possible explanation of the observed anomalies.

EXPERIMENTAL AND RESULTS

Materials and methods of assay. Substrate and enzyme preparations and the method of enzyme assay were as described in Baum *et al.* (1958). The enzyme preparation generally used was that obtained from acetone-dried human liver (preparation 1).

Influence of the reaction products on the time course of the reaction

The anomalous kinetics of human arylsulphatase *A* are apparently dependent on a slow inactivation of the enzyme in the presence of substrate and the subsequent partial restoration of enzyme activity as the reaction is allowed to proceed. It was possible that the latter phenomenon might be related to the accumulation of one or both of the products of reaction, i.e. 4-nitrocatechol and SO_4^{2-} ions. It was therefore of interest to study the influence of these products on the course of the reaction.

Effect of inorganic sulphate. Fig. 1 shows the effect of SO_4^{2-} ions on the time course of the enzymic desulphation of 5 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) by arylsulphatase *A* in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5. It will be observed that at concentrations up to 2·5 mM, SO_4^{2-} ion has little effect on the initial part of the time–activity curve (stage I, see Baum *et al.* 1958). On the other hand, SO_4^{2-} ion exerts a considerable activating effect on the later stages of the enzymic reaction. At higher concentrations of SO_4^{2-} ion, enzyme activity during stage I is inhibited appreciably but again there is marked activation during the later stages.

Fig. 2 shows the effect of 0·5 mM-Na_2SO_4 on the pH–activity curves of the enzyme when measured over varying incubation periods. The curves obtained under identical experimental conditions, but in the absence of SO_4^{2-} ions, are also shown. Although enzyme activity at pH 4·4 is only slightly affected by SO_4^{2-} ions, the latter has a considerable activating effect on the enzyme at pH 5, particularly after the first 30 min. of the reaction (i.e. during stages II and III). As a result of this activation, the pH curve, irrespective of the period of incubation, always shows two peaks of activity. By suitably modifying the experimental conditions, similar results can be obtained with

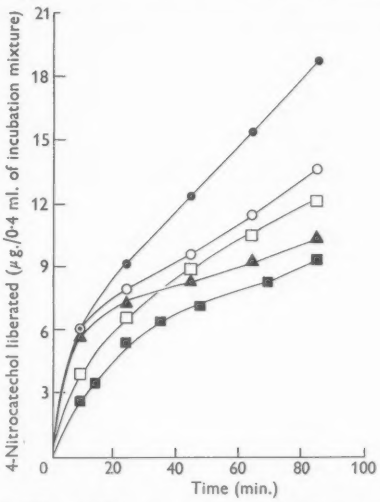


Fig. 1. Effect of SO_4^{2-} ions on the time–activity curves for human arylsulphatase *A* (ten times dilution of preparation 1) acting at 37·5° on 5 mM-NCS in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5. Concentrations of Na_2SO_4 in the incubation mixture were 0·25 mM (○), 1·25 and 2·5 mM (●), 12·5 mM (□), 25 mM (■). A control curve (▲), where no Na_2SO_4 was added, is also shown.

* Part 21: Baum, Dodgson & Spencer (1958).

potassium *p*-nitrophenyl sulphate (NPS) as the assay substrate; then the peak at pH 6·1 becomes predominant (cf. Fig. 6 of Baum *et al.* 1958).

The effect of adding SO_4^{2-} ions to the incubation mixture during stage II of the reaction is shown in Fig. 3*A*. The enzyme was allowed to act against 5 mM-NCS in 0·25 M sodium acetate–acetic acid buffer, pH 5, the concentration of enzyme used being such that approx. 7 μg. of nitrocatechol was liberated in 80 min. by 0·4 ml. of the incubation mixture. At this time, stage II of the reaction was well established and less than 5 μg. of SO_4^{2-} ion/0·4 ml. of incubation mixture had been liberated. SO_4^{2-} ion was now added to the reaction vessel (0·01 ml. of 25 mM-Na_2SO_4 soln./ml. of incubation mixture present), the concentration of SO_4^{2-} ions in the mixture thereby being increased approximately threefold. A similar volume of water was added to the control experiment. It will be seen from Fig. 3*A* that SO_4^{2-} ions caused a rapid increase in enzyme activity.

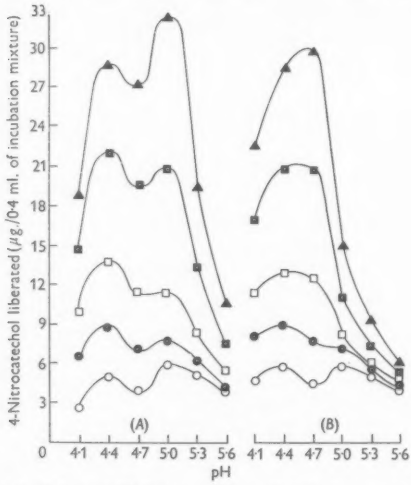


Fig. 2. Effect of SO_4^{2-} ions on the pH–activity curves for human arylsulphatase *A* plotted for various time intervals. The enzyme (ten times dilution of preparation 1) was acting on 5 mM-NCS in the presence of 0·25 M sodium acetate–acetic acid buffer at 37·5°. (*A*) pH–Activity curves in the presence of 0·5 mM-Na_2SO_4; (*B*) normal pH–activity curves. ○, 10 min.; ●, 30 min.; □, 75 min.; ■, 155 min.; ▲, 240 min.

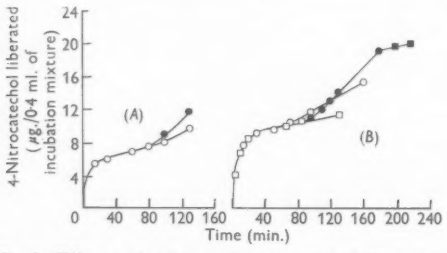


Fig. 3. Effect on the time–activity curves for human arylsulphatase *A* of Ba^{2+} and SO_4^{2-} ions when added at various stages during the reaction. (*A*) Addition of SO_4^{2-} ions during stage II of the reaction: ○, control; ●, SO_4^{2-} ions added. (*B*) Addition of Ba^{2+} ions at zero time followed by successive additions of excess of SO_4^{2-} ions and excess of Ba^{2+} ions: ○, control; □, Ba^{2+} ions added at zero time; ●, excess of SO_4^{2-} ions added; ■, excess of Ba^{2+} ions added.

These experiments suggested that the liberation of SO_4^{2-} ions during the course of the enzymic reaction could be at least partly responsible for the apparent reactivation of arylsulphatase *A* which occurs during stage III. Further support for this view was obtained by examining the effect of Ba^{2+} ions on the course of the reaction. In the presence of 3 mM-barium acetate, little or no reactivation of the enzyme occurred compared with a control experiment (Fig. 3*B*). Reactivation of the barium-treated enzyme could be achieved by adding to this incubation mixture 0·01 ml. of an aqueous solution of Na_2SO_4, the concentration of which was adjusted so as to provide a 0·6 mM excess of SO_4^{2-} ions (Fig. 3*B*). Subsequently the enzyme could again be inactivated by addition of a slight excess of barium acetate. It is clear from Fig. 3*B* that Ba^{2+} ion has little or no effect on stage I of the enzyme reaction. It will be recalled that at concentrations up to 2·5 mM, SO_4^{2-} ion was also without effect on stage I of the reaction (cf. Fig. 1). Other experiments showed that when SO_4^{2-} ion was present in the incubation mixture from the beginning of the reaction in sufficient concentrations to cause inhibition during stage I, addition of a slight excess of Ba^{2+} ions during the early stages of the reaction merely abolished the inhibition. If the Ba^{2+} ions were added during the later stages immediate inactivation resulted. It follows therefore that whatever the nature of the inactivation process, SO_4^{2-} ion does not prevent its occurrence.

Fig. 2 suggests that the effect of SO_4^{2-} ions on the course of the reaction was less marked at pH 4·4 than at 5·0. It was therefore of interest to examine the effect of Ba^{2+} ions on the time course of the reaction at pH 4·4. The results of this experiment are shown in Fig. 4, from which it can be seen that stage II was achieved more slowly at the lower pH. When the enzyme reaction was allowed to attain stage II at pH 5 in the presence of Ba^{2+} ions, readjustment of the pH of the reaction mixture from 5·0 to 4·4 at this point did not result in any immediate increase in enzyme activity. It was therefore concluded that the rate of inactivation

was greater at the higher pH but that the overall course of the reaction was the same.

Effect of sulphate in the absence of 4-nitrocatechol. It will be shown later that 4-nitrocatechol, the second reaction product, also affects the time course of the reaction. For this reason an attempt was made to confirm the effect of SO_4^{2-} ion on the inactivated enzyme in the absence of 4-nitrocatechol. Arylsulphatase *A* (0·6 ml. of preparation 1, see Baum *et al.* 1958) was incubated at 37·5° in a stoppered 15 ml. centrifuge tube for 3 min. with 0·6 ml. of 50 mM-barium acetate soln. and 2·8 ml. of water. At zero time, 2 ml. of a previously warmed 15 mM solution of NCS in 0·75 M sodium acetate–acetic acid buffer, pH 5, was added to the reaction vessel. The time course of the reaction was followed and, after 30 min., when stage II of the reaction was well established, the incubation mixture was rapidly chilled to 0°. The liberated 4-nitrocatechol was then carefully extracted (by gentle inversion of the tube) with four successive 6 ml. portions of ice-cold ether. Addition of 1 ml. of N-NaOH to a sample (0·2 ml.) of the extracted mixture showed that the liberated 4-nitrocatechol had been removed. To 4 ml. of the mixture was added 0·1 ml. of 0·2 M-Na_2SO_4, to give a slight excess of SO_4^{2-} ions, corresponding to that liberated during the enzymic reaction, and the precipitated $BaSO_4$ was removed by centrifuging. A portion (1·8 ml.) of the supernatant solution was treated with 0·01 ml. of 0·2 M-Na_2SO_4 (to give an excess of approx. mM-SO_4^{2-} ions), and to a similar portion was added 0·01 ml. of water. Both solutions were then replaced in the water bath at 37·5° and the time courses of the reactions were followed in the usual manner. The results (Fig. 5) show that SO_4^{2-} ion still caused a rapid reactivation of the enzyme when the amounts of 4-nitrocatechol present were negligible.

Effect of 4-nitrocatechol. Fig. 6 shows the effect of 4-nitrocatechol on the time course of the enzyme reaction. In concentrations up to 0·5 mM, 4-nitrocatechol has little effect on stage 1 of the reaction but causes appreciable activation during the later stages. This activation can occur in the absence of

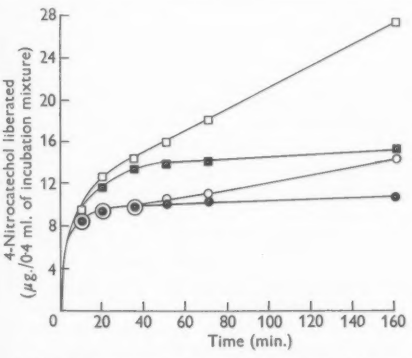


Fig. 4. Time–activity curves for human arylsulphatase *A* acting on 5 mM-NCS at 37·5° in 0·25 M sodium acetate–acetic acid buffer, pH 5·0 and 4·4, in the presence and absence of 3 mM-barium acetate. ○, pH 5 in the absence of Ba^{2+} ions; ●, pH 5 in the presence of Ba^{2+} ions; □, pH 4·4 in the absence of Ba^{2+} ions; ■, pH 4·4 in the presence of Ba^{2+} ions.

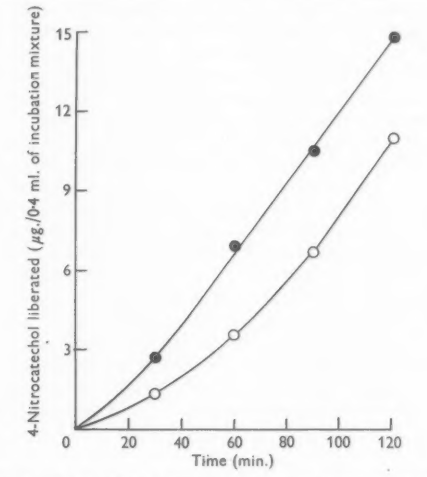


Fig. 5. Effect of SO_4^{2-} ions on the time–activity curve for 'inactivated' human arylsulphatase *A* acting on NCS. The experiment is described in detail in the text. ○, Slight excess of SO_4^{2-} ions; ●, mM-SO_4^{2-} ions.

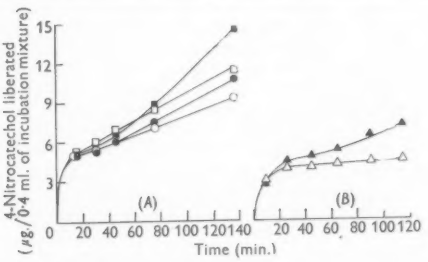


Fig. 6. Effect of 4-nitrocatechol on the time–activity curves for human arylsulphatase *A* acting on 5 mM-NCS at 37·5° in the presence of: (*A*) 0·25 M sodium acetate–acetic acid buffer, pH 5, and (*B*) 0·25 M sodium acetate, barium acetate–acetic acid buffer (3 mM-Ba^{2+} ions), pH 5. Concentrations of 4-nitrocatechol in the incubation mixtures were: (*A*) nil (○), 0·2 mM (●), 0·4 mM (□), 0·5 mM (■), and (*B*) nil (△), 0·4 mM (▲).

SO_4^{2-} ions since the effect is also observed when Ba^{2+} ion is present in the reaction mixture (Fig. 6*B*).

Effect of other compounds on the time course of the reaction

Fig. 7 shows the effect of Na_3PO_4 and $Na_4P_2O_7$ on the course of the enzyme reaction. When present in relatively high concentrations both compounds were potent inhibitors of enzyme activity. At lower concentrations they tended to inhibit during stage I of the reaction only and to activate during the later stages. Similar though less-marked results were obtained with NaF. It was possible to select concentrations of these compounds at which stage I of the reaction was virtually unaffected by their presence but activation still occurred during the later stages. Moreover, the compounds were capable of reactivating the enzyme when added to a normal enzyme–substrate mixture during stage II. It is clear that the effects of these compounds are very similar to the effect produced by SO_4^{2-} ion although quantitative differences are apparent. Thus traces of $Na_4P_2O_7$ affect the enzyme reaction markedly and, indeed, when present in concentrations of 0·1 mM, the compound apparently completely abolished the anomalous kinetics.

Fig. 7 also shows the effects of varying concentrations of urea on the time course of the reaction. Increasing concentrations of urea increased the rate of inactivation of the enzyme but did not eliminate stage III. Preincubation with urea for 20 min. before addition of substrate did not affect these results.

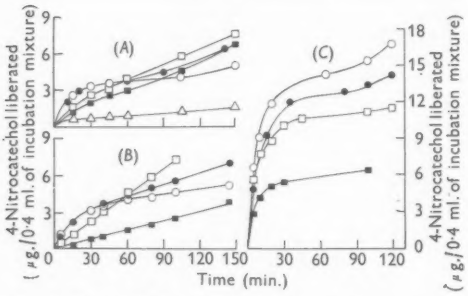


Fig. 7. Effect of PO_4^{3-} ions, $P_2O_7^{4-}$ ions and urea on the time–activity curve for human arylsulphatase *A* acting on 5 mM-NCS at 37·5° in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5. (*A*) Preparation 1 diluted 20 times and assayed in the presence of the following concentrations of Na_3PO_4: nil (○), 0·1 mM (●), 0·5 mM (□), 1·25 mM (■) and 12·5 mM (△). (*B*) Preparation 1 diluted 20 times and assayed in the presence of the following concentrations of $Na_4P_2O_7$: nil (○), 0·01 mM (●), 0·1 mM (□) and mM (■). (*C*) Preparation 1 diluted five times and assayed in the presence of the following concentrations of urea: nil (○), 6% (●), 10% (□) and 20% (■).

Attempted separation of the inactive form of the enzyme

The collective results indicated that human arylsulphatase *A* was slowly converted into an inactive form by incubation with substrate. The inactive form of the enzyme could subsequently be partially reactivated by small amounts of SO_4^{2-} ions and 4-nitrocatechol, the products of the enzymic reaction. Partial reactivation could also be achieved by the addition of small amounts of certain other compounds which, when present in higher concentrations, were potent inhibitors of enzyme activity.

Attempts were now made to separate and study the inactive form of the enzyme.

Separation by dialysis. The enzyme (1 ml. of preparation 1, see Baum *et al.* 1958) was incubated at 37·5° with 3 ml. of 10 mM-NCS in 0·5 M sodium acetate–acetic acid buffer, pH 5. After 1 hr. the incubation mixture ('inactive' preparation) was removed from the water bath and dialysed for 24 hr. against running water in order to remove unchanged NCS and the reaction products. A control experiment was made in which the buffered substrate was replaced by 0·5 M sodium acetate–acetic acid buffer, pH 5. The two diffusates were each diluted to 6 ml. with water and portions (2 ml.) were incubated at 37·5° with 2 ml. of 10 mM-NCS in 0·5 M sodium acetate–acetic acid buffer, pH 5, the time course of the reaction being followed in the usual way.

Results of a typical experiment are shown in Fig. 8, from which it can be seen that although the initial reaction rate of the 'inactive' preparation

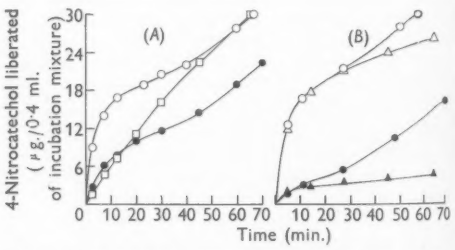


Fig. 8. Time–activity curves for preparations of the 'inactive' modification of human arylsulphatase *A*. Details of the experiments are described in the text. (*A*) Preparations subjected to the dialysis procedure; control preparation (○), 'inactive' preparation (●), 'inactive' preparation in the presence of mM-SO_4^{2-} ions (□). (*B*) Preparations subjected to the acetone-precipitation procedure: control preparation (○), 'inactive' preparation (●), control preparation in the presence of Ba^{2+} ions (△), 'inactive' preparation in the presence of Ba^{2+} ions (▲).

was less than half that of the control, the final reaction rates were very similar. This was taken to indicate that in the former preparation a considerable amount of the enzyme was already present in the inactive form and was eventually reactivated by the SO_4^{2-} ions and 4-nitrocatechol liberated as a result of the activity of the residual active enzyme. When Na_2SO_4 (final concentration, mM) was added to the reaction mixture, the final reaction rate of the 'inactive' preparation was established almost immediately. The similarity of the final reaction rates was not unexpected since the same initial concentration of enzyme was present in each case. Similar results were obtained when the initial inactivation of the enzyme was carried out in the presence of Ba^{2+} ions.

Separation by acetone precipitation. Since the dialysis procedure outlined above was not completely satisfactory for the separation of the inactive form of the enzyme an attempt was made to precipitate the inactive form with acetone. Preliminary experiments showed that the partially inactivated enzyme obtained by the procedure described in the preceding section could be precipitated by acetone (final concentration 75%, v/v) and redissolved without markedly altering the enzyme kinetics. This was a further indication that the inactive form of the enzyme was a comparatively stable entity. Attempts were therefore made to precipitate the inactive form directly from the incubation mixture.

The enzyme (1·5 ml. of preparation I, see Baum *et al.* 1958) was incubated at 37·5° with 4·5 ml. of 10 mM-NCS in 0·5M sodium acetate–acetic acid buffer, pH 5. After 45 min. the reaction mixture was rapidly cooled to 0° and treated with acetone at −5° until the concentration of acetone was 80% (v/v). The precipitate was separated by centrifuging at −2° and subsequently resuspended in 8 ml. of water ('inactive' preparation).

A control experiment was carried out in which the enzyme was incubated with buffer alone. Portions (2 ml.) of the two preparations were each incubated at 37·5° with 2 ml. of 10 mM-NCS in 0·5M sodium acetate–acetic acid buffer, pH 5, and the time courses of the reactions followed as usual (Fig. 8). Simultaneously the time courses were examined in the presence of Ba^{2+} ions in order to eliminate the effect of traces of SO_4^{2-} ion which might have coprecipitated with the enzyme during the acetone treatment (Fig. 8). The time curves obtained in the presence of Ba^{2+} ions indicate that approx. 80% of the substrate-treated enzyme had been inactivated, compared with the control. In the absence of Ba^{2+} ions the final reaction rates of the 'inactive' and control preparations were again similar although the initial reaction rate of the former was many times less than that of the control.

Reconversion of the 'inactive' enzyme into its original form

It was clear that the reactivation which occurred during stage III of the enzymic reaction could not be due to the partial re-formation of the original form of the enzyme. This follows from the fact that removal of SO_4^{2-} ions by the addition of Ba^{2+} ions during stage III resulted in an immediate loss of enzyme activity which could be restored on addition of a slight excess of SO_4^{2-} ions (see Fig. 3*B*). On the other hand, small amounts of Ba^{2+} ions, SO_4^{2-} ions and 4-nitrocatechol were without effect on stage I of the reaction (i.e. on the original form of the enzyme). This implies that stage III of the enzyme reaction represents the activity of a new form of the enzyme which is active only in the presence of 4-nitrocatechol or SO_4^{2-} ions (or certain other ions). This form of the enzyme predominates in the so-called 'inactive' enzyme preparations obtained by the dialysis or acetone-precipitation procedures.

Results obtained during the course of several preparations of the 'inactive' enzyme suggested that the dialysis procedure might be less efficient than acetone precipitation because of the tendency for some of the original enzyme to be re-formed from 'inactive' enzyme during the dialysis period. Attempts were therefore made to convert the 'inactive' enzyme back into its original form. Preliminary experiments showed that apparent reconversion of 'inactive' enzyme could be achieved by incubation with sodium acetate solutions at pH 8. Incubation with water alone was ineffective. Considerable reconversion of the 'inactive' enzyme could consistently be achieved by the procedure described below, although the extent of the reconversion varied somewhat from experiment to experiment.

A preparation of the 'inactive' form of the enzyme was made by the acetone-precipitation procedure described previously except that the precipitate was resuspended in only 4 ml. of water. A suitable control preparation was also made. A portion (1 ml.) of each preparation was treated with 0·8 ml. of M-sodium acetate, pH 8, and incubated for 2 hr. at 37·5° before allowing to stand for 18 hr. at room temperature. Subsequently, the pH of each solution was adjusted to 5 by the addition of 0·2 ml. of M-acetic acid. The time–activity curves of these solutions were then examined in the usual way after adding 2 ml. of 10 mM-NCS in 0·5M sodium acetate–acetic acid buffer, pH 5, to which had been added sufficient $BaCl_2$ to give a final concentration of 5 mM-Ba^{2+} ions. A further portion of each preparation was treated exactly as described above except that the 20 hr. incubation period in the presence of acetate at pH 8 was omitted. Ba^{2+} ion was included in all the final reaction mixtures in order to eliminate the activating effect of SO_4^{2-} ions on the 'inactive' form of the enzyme. Any activity observed therefore is largely a measure of the amount of enzyme present in the original form.

The results of these experiments are shown in Fig. 9. It will be seen that the 20 hr. incubation at pH 8 caused a slight inactivation of the control preparation (Fig. 9, curves 1 and 2), whereas the initial reaction rate of the 'inactive' enzyme preparation was increased considerably by this treatment (Fig. 9, curves 3 and 4). This indicates that 'inactive' enzyme has been reconverted into its original form.

Effect of sodium pyrophosphate on the formation of the 'inactive' enzyme

It now seemed important to consider the effect of $P_2O_7^{4-}$ ions on the formation of the 'inactive' modification of the enzyme. Fig. 7 shows that when present in concentrations greater than 0·1 mM, $P_2O_7^{4-}$ ions abolished the anomalous time–activity curve for arylsulphatase *A*. This could mean that $P_2O_7^{4-}$ ion was inhibiting the enzyme but preventing its modification to the 'inactive' form. A study was therefore made of the effect of incorporating 0·1 mM-$Na_4P_2O_7$ into the initial incubation mixture when 'inactive' enzyme was prepared by the dialysis procedure described in an earlier section. The properties of the final preparation were identical with those of the 'inactive' preparation obtained by incubating enzyme and substrate alone. Incubation of arylsulphatase *A* with acetate buffer and $P_2O_7^{4-}$ ions in the absence of substrate gave, after dialysis, an unmodified preparation with kinetics identical with those of a control from which $P_2O_7^{4-}$ ion was omitted. Whenever dialysis was carried out in the presence of $P_2O_7^{4-}$ ions it was necessary to clarify the final preparations by centrifuging. This removed a precipitate containing sufficient adsorbed $P_2O_7^{4-}$ ions markedly to affect the time–activity curves and, indeed, sometimes to eliminate the anomalies completely.

Obviously pyrophosphate does not prevent the formation of the 'inactive' modification of the enzyme. It will be recalled that similar conclusions were reached for SO_4^{2-} ions.

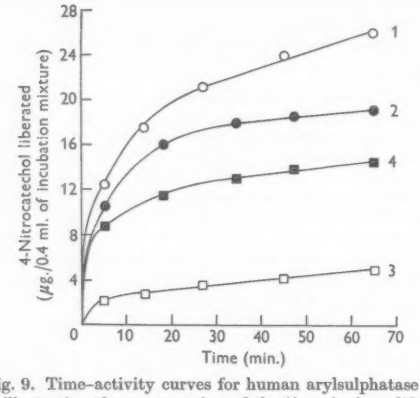


Fig. 9. Time–activity curves for human arylsulphatase *A* illustrating the reconversion of the 'inactive' modification of the enzyme into its original form. The experiment is described in detail in the text. ○, Control preparation; ●, control preparation after treatment with sodium acetate at pH 8; □, 'inactive' preparation; ■, 'inactive' preparation after treatment with sodium acetate at pH 8.

DISCUSSION

Any hypothesis about the anomalous kinetics of human arylsulphatase *A* must explain firstly the effects of temperature, pH and substrate concentration on the time course of the reaction (see Baum *et al.* 1958); and secondly, the effects of 4-nitrocatechol, SO_4^{2-} ions and certain other ions. Moreover, the hypothesis must account for the fact that it is possible to isolate an apparently modified form of the enzyme after treatment with substrate and subsequently to reconvert the modified enzyme into its original form.

A possible explanation of the anomalous time course of the enzyme reaction is that the enzyme preparation contains two distinct arylsulphatases, the more active one being rapidly but reversibly inactivated in the presence of substrate at 37·5°. Much of the kinetic data could then be explained by assuming an elaborate set of properties for these enzymes in terms of their response to temperature, pH and various ions. For example, in order to explain the fact that SO_4^{2-} ion in small amounts activates during the later stages of the reaction but is without effect during stage I, it would be necessary to assume that SO_4^{2-} ion activates the stable enzyme and inhibits the labile one in a compensating manner. The linear time–activity curve obtained when enzyme and substrate are incubated together in the presence of $Na_2P_2O_7$ (Fig. 7) would also be difficult to explain if the theory of two distinct arylsulphatases is accepted. The linearity and slope of this curve could be accounted for only by assuming either that $P_2O_7^{4-}$ ion prevents the inactivation of, but partially inhibits, the labile enzyme or that it prevents the latter from reacting with substrate in the first place. It has already been shown that modification of arylsulphatase *A* still occurs in the presence of $P_2O_7^{4-}$ ions and hence the second possibility would have to be invoked. It is difficult to visualize an inactivation of enzyme, which is known to take place only in the presence of substrate, being achieved under conditions where the enzyme is prevented from reacting with substrate. Paper electrophoresis (under varying experimental conditions) of a number of different preparations of human arylsulphatase *A* has failed to reveal the

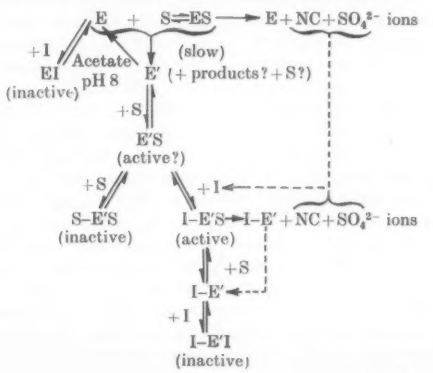
presence of a second enzyme, although this is not proof of homogeneity.

A second possibility is that the enzyme is acting in a manner analogous to that of chymotrypsin towards monopotassium *p*-nitrophenyl acetate and certain related esters. It has been shown (Macdonald & Balls, 1957; Dixon & Neurath, 1957) that, initially, the hydrolysis of these esters is accompanied by a simultaneous stoicheiometric acylation of the enzyme. This results in a rapid decrease in enzyme activity. Complete inactivation is never achieved, however, since the acylated enzyme undergoes a relatively slow spontaneous hydrolysis and this results in the establishment of a steady state of reaction which is considerably less than the initial rate. The time course of this enzyme reaction is thus, in some ways, similar to that observed for arylsulphatase *A*. When considered from a quantitative point of view, however, it is doubtful whether the mechanism of action of arylsulphatase *A* could be dependent on a sulphation of the enzyme analogous to the acylation of chymotrypsin, since it is almost certain that the initial reaction between arylsulphatase *A* and NCS is not restricted to a stoicheiometric process. Assuming a molecular weight of 10 000 for arylsulphatase *A*, then even if preparation 1 (see Baum *et al.* 1958) contained no other protein, at least 20 molecules of NCS would have been hydrolysed/molecule of enzyme during the initial reaction. Moreover, preparation 1 is by no means pure and for preparation 1*A* (see Baum *et al.* 1958) the corresponding figure is 200. Similar figures can be derived for purified preparations of ox-liver arylsulphatase *A* (see Roy, 1953). If arylsulphatase *A* was undergoing a sulphation analogous to the acylation of chymotrypsin this would therefore imply either that there were several 'active' sites per enzyme molecule or that rapid esterase activity without concomitant sulphation was proceeding at the same time. It is difficult to see how an inactive sulphated enzyme could be reactivated by SO_4^{2-} or other ions. Although a number of different nitrophenyl esters are capable of being hydrolysed by chymotrypsin, the sulphate ester is not (Macdonald & Balls, 1957). We have confirmed this observation and have made similar findings for NCS.

Other enzymes (e.g. the phenol oxidases, see Dawson & Tarpley, 1951) exhibit time–activity curves which are characterized by a short period of rapid activity followed by a reaction inactivation. However, a search of the literature has revealed no case of an enzyme having an anomalous time–activity curve which can be influenced by its reaction products in a manner analogous to that in which SO_4^{2-} ions and 4-nitrocatechol influence arylsulphatase *A*.

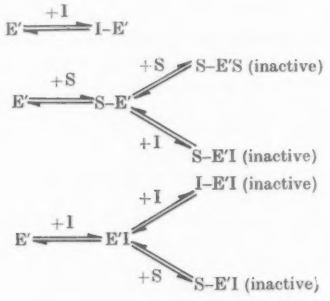
Roy (1957) has suggested that with ox arylsulphatase *A* the enzyme–substrate complex can break down not only into active enzyme plus products but also into an inactive enzyme plus products, both forms of enzyme being in equilibrium with the enzyme–substrate complex. There is an initial rapid formation of the inactive form of the enzyme, but, as the reaction products accumulate, active enzyme can be re-formed from the inactive form via the intermediary enzyme–substrate complex. However, he points out that this theory is at variance with the observation that SO_4^{2-} ion does not markedly influence the rate of initial inactivation. It is difficult to see how this theory could account for the reconversion into the original form of the 'inactive' enzyme preparation which was achieved by treatment with acetate alone during the course of the present work.

Although at this stage of the investigations any suggested explanation of the anomalous kinetics of human arylsulphatase *A* must be regarded as tentative, the following interpretation is consistent with the observed facts. During the interaction of enzyme and substrate a new substrate-binding site (there may be more than one such site per enzyme but only the simple case need be considered) is slowly exposed in the enzyme. The new site is capable of binding substrate and also the products of the enzyme reaction. Certain other compounds (PO_4^{2-}, $P_2O_7^{4-}$, F^- ions) which act as inhibitors towards the active centre of the enzyme can also bind to the new site. When substrate is bound to the new site of the modified enzyme the latter is virtually inactive. On the other hand, when the reaction products (or PO_4^{2-}, $P_2O_7^{4-}$ or F^- ions) are bound to the new site the modified enzyme is active, although the active centre may still be inhibited if these compounds are present in excess. Incubation of the modified enzyme with acetate buffer alone allows a slow rearrangement of the molecule to its original form. The proposed scheme may be represented diagrammatically as follows:



where E = the original form of the enzyme, S = substrate, E′ = modified enzyme. NC = 4-nitrocatechol, I = SO_4^{2-} ions, 4-nitrocatechol, PO_4^{3-}, $P_2O_7^{4-}$ or F^- ions. S (or I)–E′ represents binding to the new site in E′ and E′S (or I) binding to the active centre in E′.

For the purposes of clarity the following possible equilibria have been omitted from the scheme:



Although the kinetic complications inherent in such a scheme are formidable, it is nevertheless important to consider the scheme in greater detail and in relation to the experimental observations made. Thus in the scheme it is assumed that the slow formation of E′ from E in the presence of substrate is a process with an energy of activation which is greater than that of the normal esterase activity of the enzyme. The unusual behaviour of the enzyme towards variation in temperature (see Fig. 5 of Baum *et al.* 1958) can then be explained. At temperatures lower than 37·5°, although the esterase activity of E is reduced, the rate of formation of E′ (and hence the inactive S–E′S) is reduced to a still greater extent. Consequently, the activity of E is prolonged and, within limits, the overall sulphatase activity may therefore appear to increase with decrease in temperature.

The anomalous pH–activity curves obtained for the enzyme (Fig. 4 of Baum *et al.* 1958) can also be explained in terms of the proposed reaction scheme. Not only is the sulphatase activity of E affected by pH but the rate of formation of E′ (and hence the inactive S–E′S) is also a pH-dependent process, being greater, for example, at pH 5 than at 4·4 (Fig. 4). The observed sulphatase activity at a given pH will therefore depend on the relative contribution of each process at that pH, and under these circumstances the finding that the pH–activity curves obtained during stage I of the reaction show two distinct optima is not altogether unexpected. The persistence of the optimum at pH 4·4 and the disappearance of that at 5·0 as the reaction proceeds reflects the fact that the rate of formation of E′ (and hence the inactive S–E′S) is greater at the latter pH. However, other factors complicate the situation when substantial amounts of E′ and reaction products have been formed, since the effects of pH on the reactions in which E′ and products are involved must also then be considered. When the effect of pH on enzyme activity is studied with high concentrations of enzyme, appreciable quantities of the reaction products accumulate even during the initial stage of the reaction. This is reflected in the different pH–activity curves which are obtained at these higher enzyme concentrations (Fig. 4 of Baum *et al.* 1958) and which represent the sum of a number of different pH effects. When one of the reaction products (i.e. SO_4^{2-} ion) is present in the incubation mixture in appreciable amounts from the beginning of the reaction, the pH–activity curves are modified to an even greater extent (Fig. 2).

According to the proposed scheme, when the conversion of E into E′ is complete, the overall rate of reaction is governed by the competition between substrate and reaction products for the newly formed substrate-binding site which is present in E′. The relative proportions of substrate and products will thus determine the amount of E′ which is present in the I–E′S (active) form. At high substrate concentrations a high proportion of the modified enzyme will be in the S–E′S (inactive) form and this is in agreement with the observation that the substrate concentration–activity curve obtained during stage III of the enzyme reaction is typical of that for inhibition by excess of substrate (see Fig. 3 of Baum *et al.* 1958). In contrast, at high concentrations of enzyme, the reaction products will accumulate rapidly and will be present, even during the initial stages of the reaction, in concentrations sufficiently great to compete strongly with substrate for the new site in E′ immediately the latter is formed. Under these conditions the observed sulphatase activity during stage III of the reaction is relatively greater than when small amounts of enzyme are present (Fig. 1 of Baum *et al.* 1958).

Stage II of the enzyme reaction is most marked at low enzyme concentrations (Fig. 1 of Baum *et al.* 1958), and this is in accord with the reaction scheme. At low enzyme concentrations when formation of E′ is complete only small amounts of the reaction products will be present in the reaction mixture. The amount of I–E′S which can be formed at this stage will therefore be small. Nevertheless, the small amount present will constantly be forming products and the reaction at this stage becomes autocatalytic, leading eventually to stage III when a relatively high proportion of the enzyme is in the I–E′S (active) form.

From the above considerations it is clear that the presence of small amounts of SO_4^{2-} ions at the

beginning of the enzyme reaction would tend to eliminate stage II and to accentuate stage III. This is in agreement with the experimental observations (Fig. 1). However, when present in high concentrations from the beginning of the reaction, SO_4^{2-} ion competes with substrate not only for the new site in E′ but also for the active centres of both E and E′. The result of this would be inhibition during stage I and, depending on SO_4^{2-} ion concentration, reduced activation or even inhibition during the later stages (cf. Fig. 1). The activation which results from the addition of small amounts of SO_4^{2-} ion to preparations of 'inactive' enzyme (i.e. E′, see Fig. 8) or to incubation mixtures during later stages of the enzyme reaction (Fig. 3) is consistent with the proposed scheme.

If Ba^{2+} ion is present in the incubation mixture from the beginning of the reaction, then the effects due to SO_4^{2-} ions are abolished and stage II is thereby prolonged (Fig. 3). Indeed, the effects of adding Ba^{2+} ions to any reaction mixture at any stage are explicable in terms of the elimination of sulphate.

It seems likely that 4-nitrocatechol affects the enzyme reaction in a way comparable with that observed for SO_4^{2-} ions (Fig. 6). However, in view of experimental difficulties associated with the sensitivity of the assay procedure, no detailed study has been made of the effects of added 4-nitrocatechol.

The effects of PO_4^{3-}, $P_2O_7^{4-}$ and F^- ions on the enzymic reaction are apparently similar to those observed for SO_4^{2-} ions (see Fig. 7), although quantitative differences are apparent. However, the linear time–activity curves obtained when $P_2O_7^{4-}$ ion is present in the incubation mixture in concentrations greater than 0·1 mM are of considerable interest, since it is clear from the experimental results that conversion of E into E′ still occurs under these conditions. The most likely explanation of these findings which is in keeping with the proposed scheme is that the affinity of $P_2O_7^{4-}$ ion for the new site in E′ is so great that, under these conditions, S–E′S is never formed. The linearity of the time–activity curve may also mean that I–E′ has the same activity as E. The observation that the reaction rate in the presence of $P_2O_7^{4-}$ ion is less than that during stage I of the reaction in the absence of $P_2O_7^{4-}$ ion would then be interpreted as being due to the equal inhibition of the active centres of E and I–E′ by free $P_2O_7^{4-}$ ion.

Although the experimental observations are in accord with the proposed reaction scheme further work will be necessary in order to establish it conclusively. Thus the nature of changes involved in the formation of E′ and the reconversion of E′ into E are at present obscure. However, the fact that urea appears to accelerate the production of the modified enzyme (Fig. 7) may indicate that the changes involve the rupture and re-formation of hydrogen bonds. The nature of the new site which is exposed when E is converted into E′ is of some interest since it appears to be capable of binding not only substrate and SO_4^{2-} ions but also 4-nitrocatechol.

Of more immediate interest is the fact that, even after prolonged dialysis, preparations of arylsulphatase *A* to which $P_2O_7^{4-}$ ion has been added apparently retain sufficient $P_2O_7^{4-}$ ions (before centrifuging) to give linear time–activity curves. This may be of considerable significance since human-liver arylsulphatase *B* (Dodgson & Wynn, 1958), which exhibits apparently normal kinetics, is prepared from aqueous extracts of acetone-dried liver which also contain both arylsulphatase *A* and free and combined $P_2O_7^{4-}$ ions. The possibility that the two arylsulphatases might be interconvertible must therefore be considered. The properties of mixtures containing arylsulphatase *A* and $P_2O_7^{4-}$ ions are now being studied and attempts to interconvert arylsulphatases *A* and *B* are in progress.

SUMMARY

1. The anomalous time course of the reaction between human arylsulphatase *A* and dipotassium 2-hydroxy-5-nitrophenyl sulphate is markedly affected by the products of the reaction, i.e. 4-nitrocatechol and SO_4^{2-} ions. Both these products, when present in small amounts, are without effect on the initial stages of the enzyme reaction but exert an activating effect during the later stages of the reaction.

2. The effect of SO_4^{2-} ions can be abolished by addition of a slight excess of Ba^{2+} ions and subsequently restored by the further addition of a slight excess of SO_4^{2-} ions.

3. PO_4^{3-}, $P_2O_7^{4-}$ and F^- ions are similar to SO_4^{2-} ions with regard to their effect on the time course of the enzyme reaction. When present in higher concentrations, these ions inhibit enzyme activity.

4. A modified form of the enzyme can be separated from the incubation mixture at the end of the initial period of high enzyme activity. The modified form of enzyme can be reconverted into the original form by incubation with sodium acetate solution at pH 8.

5. A possible explanation of the anomalous kinetics of arylsulphatase *A* is offered in which it is suggested that the observed effects result from the slow exposure of a second substrate-binding site as the enzymic reaction proceeds. This second site can bind substrate to give a form of enzyme which is virtually inactive or it can bind SO_4^{2-}, PO_4^{3-} and $P_2O_7^{4-}$ ions and 4-nitrocatechol to give a form of enzyme which is still active. The final

rate of reaction is governed by the relative affinity of the new site for substrate or reaction products.

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Growth of *Acetobacter suboxydans* and the Oxidation of Aldoses, Related Carboxylic Acids, and Aldehydes

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Acetobacter suboxydans was first isolated by Kluyver & de Leeuw (1924) from beer. Referring to the oxidation of carbohydrates during aerobic growth, they described the organism as 'characterized by its slight intensity of oxidation which leads to an accumulation of intermediary products'. The discovery of the selective oxidation of polyols by growing cultures of *Acetobacter xylinium*, resulting in the accumulation of the corresponding ketoses, led to the enunciation of the well-known Bertrand rule that has been confirmed and extended by later workers studying *A. suboxydans*. Growth on aldoses as carbon source leads to the formation of the corresponding aldonic acids, and it is probable that gluconic acid is the initial product of the action of all *Acetobacter* species on glucose. More extensive oxidation of glucose to yield 5-oxogluconic acid during growth is a feature of a number of *Acetobacter* species, including *A. suboxydans*, and the concomitant formation of 2-oxogluconic acid by this organism has also been reported. These aspects have been reviewed recently by Rao (1957).

The oxidation of some carbohydrates by suspensions of *A. suboxydans* has been the subject of a number of investigations (Butlin, 1936, 1938*a*, *b*; Kluyver & Boezaardt, 1938; King & Cheldelin, 1952, 1953), and the present study, of which a preliminary communication has been made (Fewster, 1956), is designed to correlate and extend much of this work as well as to examine the effectiveness of various carbohydrates as energy sources for growth and how these, in turn, influence the oxidative activity of the harvested organisms. This first paper is concerned with growth of the organism and the oxidation of aldoses and a number of aldehydes and carboxylic acids.

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EXPERIMENTAL

Organism. The strain of *A. suboxydans* used was no. 621 of the American Type Culture Collection and was obtained from the National Collection of Type Cultures. It was maintained on malt wort (Walker & Tosic, 1946) containing chalk (2%, w/v; previously sterilized at 160° for 4 hr.) in suspension solidified with agar (2%, w/v) in sloped test tubes; the tubes were incubated at 30° for 48 hr.

Media

Media for growth tests. Semi-defined medium *B* (sterilized by autoclaving for 10 min. at 10 lb./in.²) contained per litre: acid-hydrolysed casein (vitamin-free), prepared by the method of Snell & Rannefeld (1945), equivalent to 6 g. of original casein; DL-tryptophan, 100 mg.; L-cysteine hydrochloride, 100 mg.; KH_2PO_4, either 10 g. (medium B_1) or 27 g. (medium B_2); $MgSO_4,7H_2O$, 400 mg.; $FeSO_4$, $(NH_4)_2SO_4,6H_2O$, 30 mg.; $MnSO_4,4H_2O$, 20 mg.; calcium pantothenate, 2 mg.; nicotinic acid, 2 mg.; *p*-aminobenzoic acid, 27·5 μg.; Tween 80 (Honeywill and Stein Ltd., London S.W. 1), 1 ml.; the medium was adjusted to pH 6 with N-NaOH. Complex medium *C* (sterilized by autoclaving for 15 min. at 10 lb./in.²) contained per litre: peptone (Evans Medical Supplies Ltd., Liverpool), 10 g.; yeast extract (Marmite Ltd., London), 10 g.; the pH of the medium was adjusted to 6 with N-NaOH.

Media for bulk growth of the organism. Two types of solid media were employed: semi-defined medium B_1 or complex medium *C* (90 ml.) was added to agar (2 g.) in a Roux bottle; the mixture was autoclaved for 15 min. at 10 lb./in.² and to it was added 10 ml. of a sterile aqueous solution (50%, w/v) of the carbohydrate under investigation. To neutralize the acids produced during growth on an aldose, sterile chalk (2 g.) was added to each bottle and suspended

evenly in the medium just before solidification. The presence of a neutralizing agent was not required when the test substance was the neutral salt of an aldonic acid, a polyol or a ketose. The organism was also grown in bulk in liquid media B_1 and C containing various carbohydrates.

Growth of the organism

For testing various carbohydrates as energy sources for growth 2·5 ml. of a solution (1 or 5%, w/v) of the sugar (autoclaved for 10 min. at 10 lb./in.^{2}) was added to 2·5 ml. of sterile basal medium in glass tubes shaped like an inverted T (Monod, Cohen-Bazire & Cohn, 1951). After inoculation with 0·1 ml./tube of a 1:100 dilution of a suspension (0·12 mg. dry wt. of cells/ml.) in sterile water of organisms from a stock slope, the tubes were attached to a rocking device (see van Heyningen & Gladstone, 1953) and shaken (36 oscillations/min.) in a water bath at 30° for periods of time up to 96 hr. The extent of growth is recorded as the galvanometer reading given by an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex) measured directly in the T-tubes, and with a neutral-density filter and the uninoculated medium to obtain the zero setting. A culture containing 0·5 mg. dry wt. of cells/ml. gave a reading of 21; there was a linear relationship between reading and dry wt. of cells up to a reading of 45.

For bulk growth of the organism on solid media each Roux bottle of media was shown with 1·5 ml. of a suspension (0·12 mg. dry wt. of cells/ml.) of cells from a stock slope, and the inverted bottles were incubated at 30° for periods of time up to 14 days. The organisms were harvested in water and concentrated by centrifuging. For bulk growth in liquid media large T-tubes each containing 25 ml. of sterile medium B_1 or C, 25 ml. of a sterile solution (5%, w/v) of the test substance and sterile chalk (0·3 g.) if required, were each inoculated with 0·5 ml. of a suspension (EEL 5) of the organism. The T-tubes were shaken in a water bath at 30° for 72 hr. and the cells harvested by centrifuging. The organisms from all types of media were washed twice by dispersion in water (a volume equal to half that of the culture medium), followed by centrifuging, and suspended finally in buffer (0·05 M) of the desired pH at a concentration of 20 mg. dry wt./ml.

Analytical methods

Chromatography. In many cases identification of the products of metabolism was by descending chromatography on Whatman no. 1 paper at room temperature, phenol–water (4:1, w/v) and the upper phase of a mixture of butan-1-ol–acetic acid–water (4:1:5, v/v) being used as general developing solvents. The two oxogluconic acids were readily separated (Table 1) by the use of (*a*) a solution of picric acid (4%, w/v) in 2-methylpropan-2-ol–water (4:1, v/v), (*b*) *iso*butyric acid saturated with water at room temperature or (*c*) propan-1-ol–formic acid–water (6:3:1, v/v); (*a*) was also used for the separation of sugar phosphates. Reducing sugars were located by spraying the dried paper with either a 1% (w/v) solution of 3:4-dinitrobenzoic acid or 2:3:5-triphenyltetrazolium chloride in 2N-Na_2CO_3 or, for chromatograms developed with the picric acid solvent, ethanolic 0·5N-NaOH, followed by heating at 100° for 3–4 min. Sugar phosphates were made visible with the ammonium molybdate reagent of Hanes & Isherwood (1949); the orcinol reagent of Klevstrand & Nordal (1950) was used for the specific detection of ketoses and an ethanolic solution of bromocresol green for non-volatile acids.

Absorption spectra. The wavelengths of the absorption bands of the cytochromes (reduced with sodium dithionite) of suspensions of the organism were measured with a Hartridge reversion spectroscope (R. and J. Beck Ltd., London W. 1), both at room temperature and after immersion of the suspensions in liquid air.

Manometry. The conventional Warburg technique was used. Each vessel contained 1 ml. of a suspension of organisms in 0·05 M-buffer (20 mg. dry wt./ml. in phosphate, pH 6, except where otherwise stated), buffer to a final concentration of 0·05 M and substrate (10–20 μmoles for measurement of the extent of the oxidation; 100 μmoles for rate determinations) was added from the side bulb after equilibration; the final volume was 3 ml. When measuring O_2 consumption the centre well of each flask contained 20% (w/v) NaOH (0·2 ml.) and a pleated filter paper; CO_2 evolution (corrected for retention by the buffer) was determined by the 'direct' method. Incubation was in air at 30°. The results of the measurements of the extent of oxidation of various substrates are expressed as moles of O_2 consumed or moles of CO_2 evolved/mole of substrate, blank values, which were very low (0·5–1·0 μl. of O_2/mg. dry wt. of cells/hr.), having been deducted.

Chemicals

Calcium 5-oxogluconate was obtained in good yield by growth of *A. suboxydans* in a yeast-extract medium containing glucose and chalk (Kulka & Walker, 1954) and removed by filtration. The crude salt was purified by dissolving in the minimum volume of 3N-HCl at 50°, the solution filtered and the filtrate adjusted to pH 6·9 with aq. 3N-NH_3 soln.; the precipitated calcium salt was removed by filtration and twice recrystallized from hot water to give pure $(C_6H_9O_7)_2Ca,2\frac{1}{2}H_2O$. A solution of 0·05 M-sodium 5-oxogluconate was prepared in the following

Table 1. *Chromatography of oxogluconic acids on paper*

Figures given are R_G values, i.e. ratio of the distance moved by the compound to the distance moved by glucose.

	Developing solvent		
Compound	Picric acid (4%) in 2-methyl-propan-2-ol–water (4:1)	*iso*Butyric acid satd. with water	Propan-1-ol–formic acid–water (6:3:1)
2-Oxogluconic acid	1·13	0·86	1·06
5-Oxogluconic acid	1·49	1·04	1·30

manner: calcium salt (117 mg.) was dissolved in 0·2 N-HCl (5 ml.) and 0·25 M-oxalic acid (1 ml.) added; the solution was adjusted to pH 6·5 with 0·4 N-NaOH, centrifuged, the precipitate washed with a little water, the washings added to the supernatant and the volume made up to 10 ml. with water. Solutions of sodium 2-oxogluconate were prepared from calcium 2-oxogluconate, $(C_6H_9O_7)_2Ca,3\frac{1}{2}H_2O$, in a similar manner; these solutions were stored at −20°. Solutions of D-glucosone were prepared by acid hydrolysis of 1:2-2:3-5:6-tri-*O*-*iso*propylidene-(2-hydroxy-D-*arabino*-hexose) (Bayne & Fewster, 1956). 3:6-Anhydro-D-glucose (m.p. 120°, uncorrected) was synthesized by the method of Ohle, Vargha & Erlbach (1928). αα-Trehalose was isolated from yeast. Grateful acknowledgement is made of the following gifts: 6-*O*-methyl-D-glucose (Dr D. J. Bell), 2-deoxy-D-glucose (Dr S. Bayne), D-glucosamine hydrochloride (2-amino-2-deoxy-D-glucose hydrochloride), *N*-acetyl-D-glucosamine and D-glucosaminic acid (Dr P. W. Kent), α- and β-methyl-D-glucopyranoside (Dr N. W. Taylor), sodium glyoxylate (Dr H. L. Kornberg) and methyl phenazonium methosulphate (Professor F. Dickens, F.R.S.).

Other compounds were commercial products; the sodium salts of carboxylic acids and sugar phosphates were used. Furfuraldehyde was purified by redistillation under reduced pressure; acetaldehyde solutions were standardized by titration with bisulphite. The sodium salt of 2:4-dinitrophenol was used for studies of inhibition.

RESULTS

Growth of the organism on various energy sources

Table 2 shows the results of growth in liquid semi-defined medium at two levels of buffering capacity (medium B_1, 0·03 M-phosphate, and B_2, 0·1 M-phosphate, both at pH 6) and in liquid complex medium *C*, each containing various carbohydrates as energy sources at two concentrations (0·5 and

Table 2. *Effectiveness of various carbohydrates as energy sources for growth in liquid media*

Incubated in rocked T-tubes at 30°; all media pH 6. Tr., Indicates 'trace' growth.

Energy source	Final concn. (%, w/v)	Medium	Growth (mg. dry wt. of organisms/100 ml.) 24 hr.	48 hr.	72 hr.	pH after incubation for 72 hr.
(*a*) D-Glucose	0·5	B_1	7	10	12	4·0
		B_2	12	35	40	5·8
		C	15	20	25	3·5
	2·5	B_1	5	10	10	3·0
		B_2	5	10	15	3·0
		C	35	40	45	3·0
(*b*) Sodium D-gluconate	0·5	*C*	2	7	20	7·0
	2·5	*C*	Tr.	Tr.	5	6·7
(*c*) D-Mannitol	0·5	B_1	Tr.	50	55	5·1
		B_2	Tr.	65	70	6·0
		C	40	55	55	4·9
	2·5	B_1	Tr.	80	95	4·7
		B_2	5	85	105	5·0
		C	80	100	105	4·8
(*d*) D-Fructose	0·5	B_1	Tr.	20	65	5·0
		B_2	Tr.	25	70	6·0
		C	Tr.	20	60	5·0
	2·5	B_1	Tr.	15	70	4·7
		B_2	Tr.	10	70	5·1
		C	Tr.	30	60	4·8
(*e*) D-Sorbitol	0·5	B_1	7	50	60	5·3
		B_2	7	65	70	5·9
		C	25	50	55	5·0
	2·5	B_1	60	90	100	5·0
		B_2	55	105	105	5·3
		C	75	95	100	5·1
(*f*) L-Sorbose	2·5	*C*	Tr.	7	12	5·4
(*g*) Glycerol	0·5	B_1	5	40	65	5·1
		B_2	5	45	70	5·9
		C	35	45	50	5·3
	2·5	B_1	10	85	85	5·1
		B_2	15	90	90	5·5
		C	60	85	95	4·8

2·5 %, w/v, final concentration). In the absence of added carbohydrate no growth was obtained in the semi-defined medium and 'trace' growth in the complex medium.

Except in medium B_2 with low sugar concentrations, growth on glucose in semi-defined medium was rapidly inhibited, as compared with that on glucose in the complex medium, by the fall in pH due to gluconic acid produced overcoming the buffering capacity of the medium. This acid arises not only as the product of the utilization of glucose during growth but also as the product of the oxidation of glucose by non-proliferating organisms, a reaction which occurs at a pH as low as 3 (see later), whereas the optimum pH for growth is 5–6. Growth on the complex medium was more rapid than on the defined medium; although after incubation for 72 hr. the pH of the medium had also fallen to 3, the overall growth was much greater. In Fig. 1 the progress of growth after a large inoculum in the liquid complex medium containing glucose is plotted together with the fall in pH, illustrating the inhibition of growth by acid production. Maximal growth with glucose occurred in media containing chalk as a neutralizing agent (Fig. 1). The results obtained with this modification in semi-defined medium B_1 and complex medium C, given in Table 3, were very similar and should be compared with the widely different results obtained for the same two media in the absence of chalk, also recorded in Table 3; the amount of chalk used per tube was such as to be completely consumed after incubation for 72 hr., which was also the time for maximum growth.

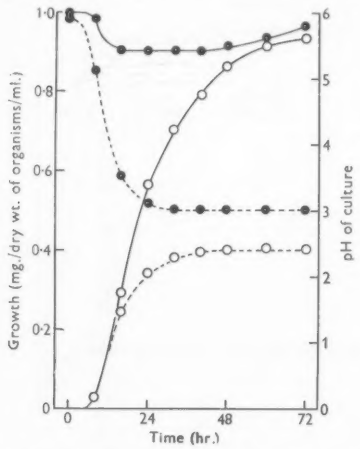

Fig. 1. Growth in liquid complex medium C containing glucose (2·5 %, w/v)±chalk (0·6 %, w/v); growth (○—○) and pH (●—●) of culture in the presence of chalk; growth (○---○) and pH (●---●) of culture in the absence of chalk. Excess of chalk and precipitated calcium salts were dissolved by the addition of HCl before measuring growth in the first experiment.

The effect of 2:4-dinitrophenol on growth with glucose was tested (Table 3); at a concentration of mM growth was inhibited by up to 90 % and at this same concentration the oxidation of glucose by suspensions of the washed organisms was inhibited by 74 % and 2- and 5-oxogluconic acids accumulated (see later, and Fewster, 1956). Considerable inhibition (65 %) of growth also occurred in the presence of 0·1 mM-dinitrophenol.

Sodium gluconate did not serve as an energy source in the semi-defined media, and poor growth was obtained in the complex medium when a rise in the pH of the medium was observed (see Table 2); the better growth at the lower concentration of sodium gluconate was inhibited by a concentration of Na^+ ions (provided by sodium chloride) equivalent to that present in the medium at the higher concentration of sodium gluconate. Fair growth was obtained with calcium gluconate (Table 3); calcium 5-oxogluconate was precipitated and was dissolved by addition of N-HCl before measurement of the extent of growth. The pH of the culture did not rise above 6·5.

Best growth of the organism was obtained on the polyols D-mannitol and D-sorbitol and was similar for all types of medium in extent, although the rate of growth on mannitol in the semi-defined media was slower than that on sorbitol; at the higher concentration good growth was also obtained on glycerol (Table 2). In all liquid media slow but good growth was obtained with D-fructose, the first product of mannitol metabolism; in contrast, growth on L-sorbose, which accumulated in good yield during growth on sorbitol, resulted only at the higher concentration and in complex medium. In neither semi-defined nor complex media was growth on mannitol significantly inhibited by 0·1 mM-dinitrophenol; a 50 % inhibition occurred with 0·2 mM-dinitrophenol (Table 3).

Growth on fructose was 60 % inhibited by 0·1 mM-dinitrophenol (i.e. very similar to the inhibition in a medium containing glucose; Table 3).

The figures for growth on solidified media containing various energy sources are given in Table 4. Again, good growth was obtained on the polyols and on glucose in the presence of chalk and was maximum after 72 hr. The reduction of growth on glucose in the absence of the neutralizing agent was again shown and was particularly apparent on the semi-defined medium B_1. Only on the complex medium C was growth on sodium gluconate obtained; with the calcium salt very variable growth was obtained on the two media. Under the conditions of this type of experiment (where a

Table 3. *Bulk growth in liquid media*

Basal medium was diluted 1:1; chalk, where included (+), 0·6% (w/v).

Energy source	Final concn. (%, w/v)	Basal medium	Chalk	DNP (final concn., mM)	Growth (mg. dry wt. of organisms/ 100 ml. in 72 hr.)	Inhibition (%)
(*a*) D-Glucose	2·5	B_1	+	.	80	.
		B_1	−	.	15	.
		B_1	−	0·1	5	65
		C	+	.	95	.
		C	+	0·1	33	65
		C	+	1·0	14	85
		C	−	.	40	.
		C	−	0·1	15	63
		C	−	1·0	4	90
(*b*) Sodium D-gluconate	0·5	B_1	−	.	0	.
		C	−	.	20	.
	2·5	B_1	−	.	0	.
		C	−	.	8	.
(*c*) Calcium D-gluconate	2·5	B_1	−	.	32	.
		C	−	.	36	.
(*d*) D-Mannitol	2·5	B_1	−	.	110	.
		B_1	−	0·1	95	14
		B_1	−	0·2	50	55
		C	−	.	120	.
		C	−	0·1	120	0
		C	−	0·2	60	50
(*e*) D-Fructose	2·5	B_1	−	.	65	.
		B_1	−	0·1	29	55
		C	−	.	55	.
		C	−	0·1	22	60

Table 4. *Bulk growth on solid media*

Energy source (5%, w/v)	Basal medium	Chalk (2%, w/v)	Growth (mg. dry wt. of organisms/ 100 ml. of medium in 72 hr.)
(*a*) D-Glucose	B_1	+	125
	B_1	−	15
	C	+	175
	C	−	50
(*b*) Sodium D-gluconate	B_1	−	0
	C	−	20–40
(*c*) Calcium D-gluconate	B_1	−	40–75
	C	−	50–100
(*d*) D-Mannitol	B_1	−	190
	C	−	240
(*e*) D-Fructose	B_1	−	40
	C	−	55
(*f*) D-Sorbitol	B_1	−	200
	C	−	215
(*g*) L-Sorbose	B_1	−	5
	C	−	30
(*h*) Glycerol	B_1	−	115
	C	−	140
(*i*) D-Galactose	C	+	20
(*j*) D-Mannose	C	+	10
(*k*) D-Xylose	C	+	20
(*l*) L-Arabinose	C	+	10
(*m*) Sucrose	C	+	5

large inoculum was used) some growth was obtained on sorbose as well as on fructose although growth on the latter was poor compared with that resulting in liquid media. Very poor growth occurred on D-galactose, D-xylose and L-arabinose; D-mannose and sucrose did not support growth. Growth on solidified media was the principal method used for the production of organisms for the testing of their oxidative capabilities in washed suspension.

Oxidation of glucose and gluconic acids by washed organisms

The results presented in this section were obtained with organisms harvested after growth for 72 hr. on solidified complex medium *C* containing glucose and chalk, and suspended in phosphate buffer, pH 6, at 30°. Glucose was extensively oxidized with an uptake of 3·75 moles of O_2 (see Fig. 2) and an output of up to 4·0 moles of CO_2/mole of glucose; these figures were constant over a wide range (1–20 μmoles of glucose/20 mg. dry wt. of cells) of substrate concentration and represent approx. 60 % complete oxidation of the molecule.

Gluconic acid, either in the form of glucono-δ-lactone or the calcium or sodium salt, was oxidized at a rate and to an extent with regard to both O_2 consumption and CO_2 evolution consistent with it being the primary product of the oxidation of glucose (Fig. 2). Chromatographic examination on paper of the contents of manometer vessels 5 min. after the addition of glucose to the suspension of organisms showed the presence of 2- and 5-oxogluconic acid as well as gluconic acid, i.e. the same products as accumulate during growth of the organism on a medium containing glucose; the formation of sugar phosphates could not be demonstrated. Under the conditions of the experiments with washed organisms, namely, low concentration of substrate, high concentration of organisms and vigorous aeration, the keto acids were rapidly further metabolized. Fresh solutions of sodium 5-oxogluconate were not oxidized by suspensions of well-washed organisms. With solutions that had been stored for several days at room temperature, or suspensions of organisms contaminated with traces of oxidizable material from the growth medium, figures for O_2 uptake of up to 2 moles and for CO_2 output of 2 moles/mole of keto acid were recorded; the latter type of oxidation was abolished by incubation of the suspension for 1 hr. at 30° in phosphate buffer, pH 6, before use. Oxidation of fresh solutions of 5-oxogluconate was not initiated by addition of di- or tri-phosphopyridine nucleotide (DPN or TPN) even at substrate concentration, adenosine triphosphate (ATP) and Mg^{2+} ions, or methyl phenazonium methosulphate or by previous incubation of the suspension with small amounts of readily oxidized substrates. However, either simultaneous addition of the keto acid and a readily oxidized substrate (such as glucose, gluconate, mannitol, fructose or pyruvate), or addition of the 5-oxogluconate during the oxidation of the other substrate, resulted in steady oxidation of the acid (Fig. 2 and Table 5); thus in 4 hr. and in the presence of 5 μmoles of glucose an O_2 uptake of 3·0 moles and a CO_2 output of 3·5 moles/mole of 5-oxogluconate (10 μmoles) was observed, O_2 consumption ceasing at a figure of 3·5 moles/mole after 5–6 hr. (corresponding values for the added glucose are subtracted). Relatively small amounts of added substrate were required to effect the oxidation of a given amount of the acid; however, a minimal requirement in terms of absolute uptake of O_2 by the added substrate for maximal oxidation of 10 μmoles of the keto acid could be measured as shown in Table 5, in which the effectiveness of glucose (oxidized to the extent of 3·75 moles of O_2/mole) is compared with that of pyruvate (0·5 mole/mole).

Sodium 2-oxogluconate was oxidized to the extent of 0·5 mole of O_2/mole (Fig. 2), with an accompanying evolution of 0·5 mole of CO_2. This degree of oxidation was not increased by addition

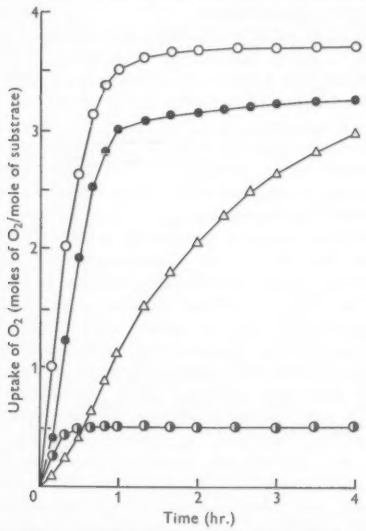

Fig. 2. Oxidation of glucose and gluconic acids (10 μmoles) by washed organisms (20 mg. dry wt. of cells) harvested after growth for 72 hr. on solidified complex medium *C* containing glucose and chalk; ○, glucose; ●, sodium gluconate; ◑, sodium 2-oxogluconate; △, sodium 5-oxogluconate plus glucose (5 μmoles), value for glucose subtracted.

of DPN, TPN, ATP and Mg^{2+} ions, or readily oxidized substrates. The non-reducing acidic product of this oxidative decarboxylation (the intermediate formation of D-arabinose could not be demonstrated) was identified chromatographically as D-arabonic acid. No evidence was obtained for the formation of 2:5-dioxogluconate shown by Katznelson, Tanenbaum & Tatum (1953) to be the product of the oxidation of 2-oxogluconate by 'aged' preparations of *Acetobacter melanogenum*.

Table 5. *Oxidation of 5-oxogluconate in the presence of added substrates*

5-Oxogluconate (10 μmoles) was added to a suspension in 0·05 M-phosphate buffer, pH 6, of washed organisms (20 mg. dry wt.) harvested after growth for 72 hr. on solidified complex medium *C* containing glucose and chalk; supplementary substrates were added simultaneously; 20% (w/v) NaOH (0·2 ml.) and pleated filter paper were placed in each centre well; total volume 3 ml.; incubated in air at 30° for 4 hr.

Added substrate (μmoles)		Uptake of O_2 by added substrate (μl.)	Uptake of O_2 by 5-oxogluconate (μl.)
None		—	0–20
D-Glucose,	5	415	670
	2	165	650
	1	83	630
	0·5	42	630
	0·1	8	170–425
Pyruvate,	10	110	630
	5	58	615
	1	10	10

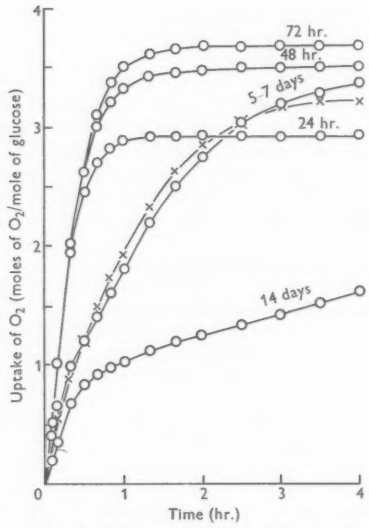

Fig. 3. Oxidation of glucose by washed organisms harvested from solidified complex medium *C* containing glucose and chalk after various incubation times; ○, incubation times as indicated; ×, oxidation of glucose by organisms harvested after incubation for 3 days followed by storage at 0–4° in sterile saline for 48 hr.

Factors affecting the oxidation of glucose

Age of organisms and pH *of growth medium.* Maximum oxidation of glucose was obtained with organisms harvested after 72 hr. from solidified complex medium *C* containing glucose and chalk; for this medium such an incubation time also gave the maximum yield of organisms (Table 4). Organisms harvested after 48 hr. were slightly less active, and very young cells (24 hr.) attacked glucose at a similar rate but to a more limited extent (Fig. 3). Periods of incubation longer than 72 hr. caused a decrease in rate (5–7 days) and later (14 days) both rate and extent of oxidation (Fig. 3). The ageing of organisms harvested after 72 hr. by storage in sterile saline for 48 hr. at 0–4° (Fig. 3) or 24 hr. in phosphate buffer, pH 6, at 30° (Fig. 5), led to a small loss of activity; rigorous washing of the organisms (dispersion in water followed by centrifuging, repeated six times) or pre-incubation in phosphate buffer, pH 6, for 2 hr. at 30° caused a slight decrease in the rate but not in the extent of oxidation.

The absence of chalk from the growth medium caused a rapid fall in pH to 3 after 72 hr. (as compared with a figure of 5·5 when the neutralizing agent was present). Under these conditions there was a progressive loss of oxidizing power of the organisms towards glucose (Fig. 4). Thus organisms harvested after incubation for 24 hr. oxidized glucose at a rate and to an extent similar to those measured for organisms grown for the same time period on a medium containing chalk; after incubation for 48 hr. the development of acidity resulted in a very considerable decrease in activity. A 72 hr. culture oxidized glucose to gluconic acid quantitatively with an uptake of 0·5 mole of O_2/mole and no CO_2 evolution; gluconic acid was not attacked. The only other substrate of the many tested that was oxidized at a comparable rate (95%) was 2-deoxy-D-glucose, to give what was tentatively identified as 2-deoxy-D-gluconic acid (Fig. 4). D-Mannose, D-xylose and L-arabinose were oxidized at 1–2% of the oxidation rate of glucose and after 4 hr. the O_2 consumed was approx. 0·05 mole of O_2/mole; all other compounds listed in Table 8 as well as D-mannitol, D-fructose, D-sorbitol, L-sorbose, D-arabitol, ribitol, glycerol and ethanol were inactive as substrates. Similar losses in oxidizing activity occurred when organisms harvested from medium containing glucose and chalk were incubated in phthalate buffer, pH 3, at

30° (Fig. 5). These results confirm the work of Butlin (1936, 1938*a*, *b*), who first demonstrated the limited oxidizing power of *A. suboxydans* towards glucose when grown under conditions where production of acid was not controlled or when normal organisms (which in Butlin's work were much less active than those that have been described in the present study) were treated with acid. In agreement with Butlin it was noted that fully active organisms were pink in colour, whereas those grown in the absence of chalk were 'dirty white'. The active pink organisms, harvested after growth for 24, 48, or 72 hr., were rich in a cytochrome component having an α-band at 552 mμ and a β-band at 524 mμ when measured after immersion in liquid air. This 'single' cytochrome was described by Smith (1954*a*, *b*) as being characteristic of *A. suboxydans* but has recently been shown to contain at least three components (Keilin & Smith, 1957). With regard to cytochrome content and appearance as well as oxidative activity, organisms harvested after growth for 24 hr. on the medium containing glucose and no chalk were indistinguishable from those grown in the presence of chalk; after growth for 48 hr. in the absence of the neutralizing agent there was a large decrease in content of the 'characteristic' cytochrome, whereas after incubation for 72 hr. the organisms contained as the only cytochrome-like constituent a small amount of material exhibiting absorption bands at 559 and 530 mμ, and were themselves very pale cream and shrunken in appearance. Thus the decrease in oxidizing capacity towards glucose with increase in incubation time in the absence of chalk, i.e. increase in acidity of the growth medium, was paralleled by either a loss or a non-production of cytochrome. These results are in contrast with those obtained when organisms grown on glucose in the presence of chalk were incubated at pH 3; under these conditions, although there was a loss of oxidizing activity (Fig. 5) and bleaching of the organisms, the presence of the characteristic cytochrome after reduction with dithionite was demonstrated in apparently undiminished amount and shown to be oxidized by O_2; a reduced cytochrome band at 559 mμ was not detected.

The activity towards glucose of organisms grown on glucose in the absence of chalk for 72 hr. could not be increased in either rate or extent by adding DPN, TPN, coenzyme A, cytochrome *c*, ATP and Mg^{2+} ions, methyl phenazonium methosulphate or

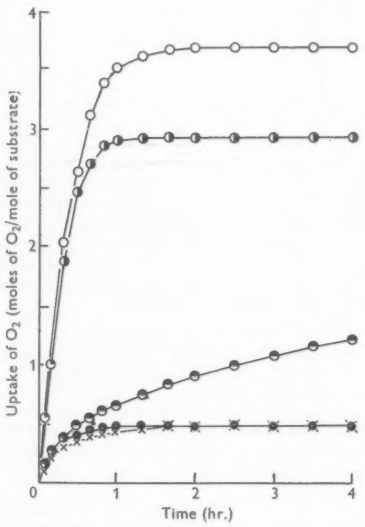

Fig. 4. Oxidation of substrates by washed organisms harvested from solidified complex medium *C* containing glucose but no chalk after various incubation times; ◑, glucose, 24 hr.; ◓, glucose, 48 hr.; ●, glucose, 72 hr.; ×, 2-deoxy-glucose, 72 hr.; ○, maximum oxidation of glucose, i.e. by organisms harvested after growth for 72 hr. on medium *C* containing glucose and chalk.

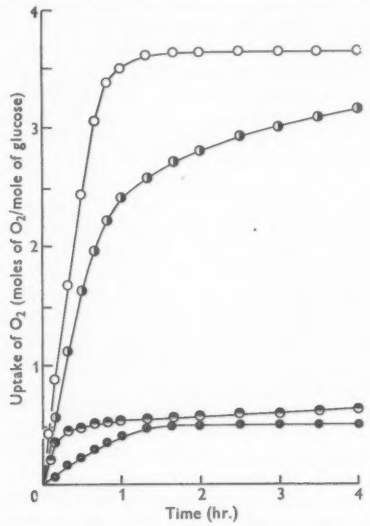

Fig. 5. Effect on the oxidation of glucose of pre-incubating organisms harvested after growth for 72 hr. on solidified complex medium *C* containing glucose and chalk; after pre-incubation the organisms were washed and resuspended in 0·05 M-phosphate buffer, pH 6; ○, control, no pre-incubation; ◑, after pre-incubation in phosphate buffer, pH 6, for 24 hr. at 30°; ◓, after pre-incubation in phthalate buffer, pH 3, for 3 hr. at 30°; ●, after pre-incubation at pH 3 for 24 hr. at 30°.

a hot-water extract of organisms harvested from a medium containing glucose and chalk.

With the reagent of Hestrin (1949) it was shown that the immediate product of the oxidation of glucose by the cytochrome-deficient organisms formed an acylhydroxamic acid; with bicarbonate buffer and a gas phase of 100 % CO_2 the presence in suspensions of the organisms of an enzyme which slowly delactonized D-glucono-δ-lactone was demonstrated manometrically. Such an enzyme was also present in much higher concentration in organisms grown in the presence of chalk. Both types of organism were also shown to be rich in catalase, but with those grown in the absence of chalk no evidence was obtained for the formation of hydrogen peroxide when glucose was oxidized to gluconic acid. Thus the O_2 uptake of 0·5 mole/mole of glucose was not increased in extent by either mM-KCN or 20 mM-hydroxylamine, both potent inhibitors of catalase; in fact the rate of oxidation was 65 and 95 % inhibited by 1 and 5 mM-KCN respectively. In addition, during the oxidation of glucose no coupled oxidation of ethanol could be demonstrated, a reaction shown to occur with notatin in the presence of catalase by Keilin & Hartree (1948*b*). Phenolindo-2:6-dichlorophenol served as a hydrogen acceptor from glucose under anaerobic conditions (demonstrated by the Thunberg technique), whereas methylene blue and 2:3:5-triphenyltetrazolium chloride were inactive.

pH *of suspension medium.* The optimum pH for the extensive oxidation of glucose by organisms harvested from a medium containing glucose and chalk was 6·0–6·5; above and below this range the extent of O_2 consumption rapidly decreased, although the oxidation of glucose to gluconic acid occurred at such extremes of pH as 3 and 9.

Growth medium with particular reference to energy source. The oxidation of glucose by washed organisms harvested after growth for 72 hr. on the same solidified complex medium *C* but containing D-galactose plus chalk, D-mannitol, D-sorbitol, D-fructose or L-sorbose instead of glucose plus chalk was similar, in both extent and rate of O_2 uptake. However, yields of organisms obtained for the various sugars differed considerably (Table 4). Organisms grown on solidified medium *C* containing sodium gluconate, or L-arabinose and chalk, oxidized glucose at a similar rate but to a decreased extent. The use of glycerol as an energy source in the same basal medium produced organisms which oxidized glucose to a greatly decreased extent (Fig. 6); gluconate was not oxidized in a manner compatible with it being the sole primary product of glucose utilization, and the extent of oxidation of the keto acids was much decreased compared with that by organisms grown in the presence of glucose (Table 6). A significant decrease in the extent of oxidation of glucose also occurred when organisms were grown on solidified semi-defined medium B_1 containing glucose and chalk (Fig. 6) or sorbitol as energy source, but this was not observed for mannitol or fructose; no growth was obtained on gluconate in this medium, and growth on sorbose was very poor (see Table 4). Maximum activity was obtained with organisms harvested from liquid complex medium *C* containing either glucose and chalk or mannitol; a decrease in activity resulted

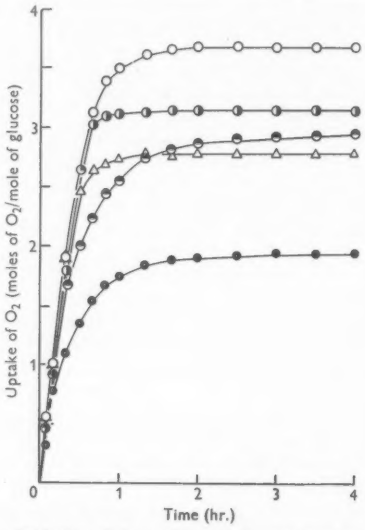

Fig. 6. Oxidation of glucose by washed organisms harvested after growth for 72 hr. on solidified media containing various energy sources; ○, organisms harvested from complex medium *C* containing glucose and chalk; ◓, from semi-defined medium B_1 containing glucose and chalk; ◑, from medium *C* containing sodium gluconate; ●, from medium *C* containing glycerol; △, from medium *C* containing L-arabinose and chalk.

Table 6. *Oxidative activity of organisms grown in the presence of glycerol*

Substrates (10 μmoles) were added to washed organisms (20 mg. dry wt.) harvested after growth for 72 hr. on solidified complex medium *C* containing glycerol (5 %, w/v) and suspended in 0·05 M-phosphate buffer, pH 6; incubated in air at 30° for 4 hr.

Substrate	Uptake of O_2 (mole of O_2/mole)	Output of CO_2 (mole of CO_2/mole)
Glucose	1·95	1·35
Gluconate	0·85	0·2
2-Oxogluconate	0·06	—
5-Oxogluconate	0·02	—
5-Oxogluconate + glucose (2 μmoles)	0·63	—

Table 7. *Effect of various energy sources for growth on the extent of oxidation of glucose by the harvested organisms*

Energy source was at 5% (w/v) and chalk, where included (+), at 2% (w/v) in solidified media; energy source was at 2·5% (w/v) and chalk, where included, at 0·6% (w/v) in liquid media; organisms were harvested after incubation for 72 hr.; glucose (10 μmoles) was added to washed organisms (20 mg. dry wt.) in 0·05M-phosphate buffer, pH 6, at 30°; figures in parentheses are times (hr.) for completion of O_2 uptake.

		Uptake of O_2 (μmoles/mole of glucose)			
Basal growth medium		*C*	B_1	*C*	B_1
Energy source	Chalk	(solidified)	(solidified)	(liquid)	(liquid)
D-Glucose	–	0·5 (1)	0·5 (1)	0·5 (1)	0·5 (1)
D-Glucose	+	3·75 (1·5)	3·0 (3)	3·5 (1·5)	3·0 (3)
Sodium D-gluconate	–	3·1 (1·5)	.	.	.
D-Galactose	+	3·7 (1·5)	.	.	.
L-Arabinose	+	2·8 (1·5)	.	.	.
D-Mannitol	–	3·55 (1·5)	3·6 (1·5)	3·6 (1·5)	3·1 (2)
D-Fructose	–	3·75 (1·5)	3·7 (1·5)	.	.
D-Sorbitol	–	3·7 (1·5)	3·1 (2)	.	.
L-Sorbose	–	3·75 (1·5)	.	.	.
Glycerol	–	1·95 (2·5)	.	.	.

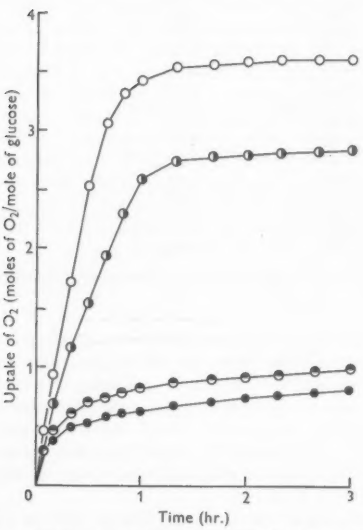

Fig. 7. Inhibition by dinitrophenol (DNP) of the oxidation of glucose at pH 6 by washed organisms harvested after growth for 72 hr. on solidified complex medium *C* containing glucose and chalk; ○, ±0·01 mM-DNP; ◑, +0·1 mM-DNP; ◓, +mM-DNP; ●, +10 mM-DNP.

with these two energy sources in liquid semi-defined medium B_1. Organisms grown in liquid complex medium *C* containing mannitol and 0·2 mM-dinitrophenol (the latter reagent caused 50% inhibition of growth—see Table 3) possessed normal activity for the oxidation of glucose. In no case was the activity towards glucose reduced by pre-incubation of the organisms in phosphate buffer, pH 6, for 1 hr. at 30°. These results are summarized in Table 7.

Chemical inhibitors. Organisms harvested after incubation for 72 hr. from solidified complex medium *C* containing glucose and chalk, and suspended in phosphate buffer, pH 6, were used; inhibitors were added to the suspension of organisms. Oxidation of glucose was 80% inhibited and that of 2-oxogluconate and 5-oxogluconate oxidation abolished by mM-KCN and 50 mM-KF; mM-arsenite caused up to 20% inhibition of the extent of glucose oxidation. The effect of various concentrations of dinitrophenol, an uncoupler of oxidative phosphorylation, is illustrated in Fig. 7; mM-dinitrophenol caused the accumulation of 2- and 5-oxogluconic acids (identified chromatographically), in approximately equal proportions, as the only detectable products of the oxidation of glucose or gluconate. A similar inhibition (73%) and accumulation was given by 0·2 mM-phenylmercury acetate, a general inhibitor of enzymes depending for their activity on the presence of free sulphydryl groups. With 0·1 mM-dinitrophenol, which inhibited growth in media containing glucose by 65%, the extent of glucose oxidation was inhibited by up to 20% (Fig. 7).

Oxidation of derivatives of D-*glucose and other aldoses*

Slight modification in the glucose molecule led to a great reduction in the degree of oxidation by organisms harvested from solified complex medium *C* containing glucose and chalk. Thus with 2-deoxy-D-glucose and 6-*O*-methyl-D-glucose O_2 was taken up rapidly to a level corresponding to the formation of aldonic acids; thereafter the uptake was very slow and ceased at a value approaching

Table 8. *Oxidation of various aldoses, disaccharides, aldehydes and carboxylic acids*

Substrates (10–20 μmoles) were added to washed organisms (20 mg. dry wt.), harvested after growth for 72 hr. on solidified complex medium *C* containing glucose and chalk, suspended in 0·05 M-phosphate buffer, pH 6; incubated in air at 30°. The following were not oxidized to a significant extent: α-methyl-D-glucopyranoside, *N*-acetyl-D-glucosamine, D-glucurone, D-glucose 6-phosphate, L-rhamnose, D-arabonate, αα-trehalose, lactose, maltose, acetate, succinate, citrate, 2-oxoglutarate, oxalate, D-tartrate, L-tartrate, *meso*tartrate, dihydroxytartrate, tartronate and glyoxylate.

Substrate	Uptake of O_2 (moles of O_2/mole of substrate) 1 hr.	4 hr.
D-Glucose	3·5	3·75
D-Gluconate	3·05	3·2
2-Oxo-D-gluconate	0·5	0·5
5-Oxo-D-gluconate (+D-glucose)	1·15	3·0
2-Deoxy-D-glucose	0·65	1·02
3-*O*-Methyl-D-glucose	0·33	0·69
6-*O*-Methyl-D-glucose	0·64 (0·85)*	0·75 (1·00)
3:6-Anhydro-D-glucose	0·03	0·15
β-Methyl-D-glucopyranoside	0·14	0·35
D-Glucosamine	0·18	0·61
D-Glucosaminate	0·06	0·18
D-Glucosone	0·41	0·83
D-Mannose	0·35	0·95
D-Galactose	0·45	0·51
D-Arabinose	0·18	0·47
L-Arabinose	0·51	0·52
D-Xylose	0·48	0·50
L-Xylose	0·11	0·25
D-Ribose	0·10 (0·55)	0·49 (1·00)
D-Lyxose	0·42	0·61
Sucrose	0·38	1·75
Acetaldehyde	0·48	0·49
Glycollaldehyde	0·48	0·51
Furfuraldehyde	0·51	0·52
Lactate	0·56	1·00
Pyruvate	0·49	0·51
Glycollate	0·05	0·09

* Figures in parentheses were obtained with organisms harvested from solidified medium *C* containing mannitol.

Table 9. *Rates of oxidation of aldoses and of gluconate*

Substrates (100 μmoles) were added to washed organisms (1·5 mg. dry wt.) harvested after growth for 72 hr. on solidified complex medium *C* containing glucose and chalk, suspended in 0·05 M-phosphate buffer, pH 6; incubated in air at 30° for 1 hr.; Q_{O_2} values (μl. of O_2 taken up/mg. dry wt. of organisms/hr.) were calculated from O_2 uptake measured during period 10–40 min. after addition of each substrate.

Substrate	Q_{O_2}
D-Glucose	750
Sodium D-gluconate	80
D-Galactose	65
D-Mannose	50
L-Arabinose	70
D-Xylose	160

1 mole of O_2/mole. D-Glucosamine and D-glucosaminic acid were slowly oxidized; *N*-acetyl-D-glucosamine was not attacked. β-Methyl-D-glucopyranoside, but not the α-isomer, was oxidized slowly and steadily. 3-*O*-Methyl-D-glucose and D-glucosone were slowly oxidized with an uptake approaching 1 mole of O_2/mole in 4 hr. 3:6-Anhydro-D-glucose was metabolized very slowly, whereas D-glucurone and D-glucose 6-phosphate were inert. Similar results were obtained with organisms harvested from solidified complex medium *C* containing mannitol, except that the oxidation of 6-*O*-methyl-D-glucose was appreciably faster (see Table 8).

D-Mannose was oxidized slowly with an uptake of 1 mole of O_2/mole and D-galactose was oxidized to D-galactonic acid, a degree of oxidation not exceeded even by organisms harvested from solidified complex medium *C* containing D-galactose and chalk. Of the aldopentoses tested, L-arabinose, D-xylose and D-lyxose were oxidized rapidly, with the consumption of 0·5 mole of O_2/mole, whereas D-arabinose, L-xylose and D-ribose were slowly oxidized to the same extent; L-rhamnose (6-deoxy-L-mannose) was not oxidized. Similar results were obtained with organisms harvested from growth medium containing mannitol, except that D-ribose was steadily oxidized with an uptake of 1 mole of O_2/mole.

D-Glucose was the most rapidly as well as the most extensively oxidized of the aldoses tested; Q_{O_2} values of a number of aldoses and of gluconate, recorded in Table 9, were determined with a concentration of organisms equal to that present in a liquid culture at half-growth (0·5 mg. dry wt./ml.).

Sucrose was the only disaccharide of those tested to be oxidized; the steady evolution of CO_2 which accompanied consumption of O_2 suggested that the sucrose was slowly hydrolysed and the resulting glucose and fructose were oxidized normally.

Oxidation of aldehydes and carboxylic acids

Organisms were harvested from complex medium *C* containing glucose and chalk; the use of mannitol as an energy source for growth did not affect the activity towards these substrates. Acetaldehyde, glycollaldehyde and furfuraldehyde were oxidized with the uptake of 0·5 mole of O_2/mole and the production of one equivalent of acid. An uptake of 1 mole of O_2/mole of lactate accompanied by an output of 1 mole of CO_2 was observed; the corresponding figures for pyruvate were 0·5 mole of O_2 and 1·0 mole of CO_2. King & Cheldelin (1954) have demonstrated the presence of a yeast-type pyruvic carboxylase in cell-free extracts of *A. suboxydans*. In agreement with King & Cheldelin (1953) it was observed that the ultimate product of pyruvate oxidation, acetate, was not oxidized and nor were a number of intermediates of the tricarboxylic acid cycle, namely, succinate, citrate and 2-oxoglutarate. L-Tartrate, oxalate and glycollate, reported to be products of the oxidation of 5-oxogluconate by various *Acetobacter* species and *Pseudomonas fluorescens* (Jackson, Koepsell, Lockwood, Nelson & Stodola, 1949), were not oxidized by washed organisms; D-tartrate, *meso*tartrate, dihydroxytartrate, tartronate and glyoxylate were also not attacked.

These results are summarized in Table 8, together with those obtained for the various aldoses.

DISCUSSION

The growth experiments show clearly that a number of polyols were satisfactory as energy sources for growth of the organism, but of the aldoses tested only glucose, in the presence of an adequate neutralizing agent, allowed good growth. The primary products of oxidation by the organism of these substances also varied considerably in their ability themselves to support growth.

The poor growth on gluconate supports the view of Stubbs, Lockwood, Roe, Tabenkin & Ward (1940) that the energy for growth on glucose is derived mainly from the oxidation to gluconic acid, the further oxidation of this acid to a mixture of keto acids being carried out by non-proliferating organisms. Fair but slow growth was obtained with fructose, the primary product of the oxidation of mannitol. Dinitrophenol at a concentration of 0·1 mM inhibited growth on glucose and on fructose by 60%, suggesting that energy-transfer mechanisms common to growth on both these sugars are inhibited. The same concentration of reagent did not significantly diminish growth on mannitol. Similar lack of inhibition of growth on sorbitol and glycerol was reported by King & Cheldelin (1953), but their study of the effect of this inhibitor was, of necessity, incomplete since, like the present author, they found sorbose (and also dihydroxyacetone) to be valueless as a promoter of growth.

All the compounds that served as good energy sources for growth were extensively oxidized by washed organisms. However, the converse was not true; of the poor promoters of growth galactose, xylose and arabinose were not oxidized beyond the stage of the corresponding aldonic acids, but gluconate and sorbose were apparently extensively oxidized as intermediates in the oxidation of glucose and sorbitol respectively. The low rate of oxidation of gluconate compared with that of glucose by washed organisms may explain the inadequacy of this compound as a growth promoter; it also provides a reason for the accumulation of primary products during growth on glucose, the rate of oxidation of gluconate being the limiting factor in the rate of formation of 2- and 5-oxogluconate. The extent of oxidation of sorbose as such (2–3 moles of oxygen/mole) by washed organisms was more limited than that of fructose (up to 4·5 moles of oxygen/mole); was greatly decreased by previous starvation of the washed organisms (unlike that of fructose); and was very much more sensitive to dinitrophenol than was that of fructose (J. A. Fewster, unpublished work).

The so-called 'energy source' may be called upon to meet requirements not only for energy but also for part at least of the available carbon for biosynthetic purposes; it is likely therefore that (*a*) the compound must be readily oxidized and (*b*) either it or one of the primary products of its oxidation may be able to enter metabolic pathways leading to biosynthesis only in a phosphorylated form. The high yields of primary products accumulating during growth of the organism suggest that in (*a*) the rate of oxidation may be more important than the extent, that the oxidative reaction is the predominant one, and that the quantitative requirement for (*b*) is small. Glucose and fructose have been shown to be readily phosphorylated by ultrasonic extracts (Fewster, 1957*b*). No evidence has been obtained for the phosphorylation of mannitol, sorbitol or sorbose; however, the first is readily oxidized to fructose and there is indirect

evidence (Arcus & Edson, 1956; Cummins, King & Cheldelin, 1957; J. A. Fewster, unpublished work) that some fructose, presumably sufficient to satisfy the need for phosphorylated intermediates, is formed from sorbitol during growth. The inadequacy of sorbose as a growth promoter may be ascribed to non-phosphorylation or a low rate of oxidation or both; these same explanations also apply to galactose, xylose and arabinose. Mannose, although phosphorylated by extracts (Fewster, 1957*b*), was also oxidized very slowly by washed organisms; an alternative explanation is that the product of mannose phosphorylation is not further utilizable by the organism. The limited growth obtained in complex medium in the presence of these sugars was probably due to the 'sparking' action of traces of a utilizable carbon source in the yeast extract. Thus any carbon compound suitable for the growth of *A. suboxydans* (e.g. glucose, glycerol or sorbitol) has been shown to be capable at the 0·1% (w/v) level of 'sparking' growth in a medium containing ethanol, erythritol or inositol, themselves unable to support growth (Fulmer & Underkofler, 1947; Rao & Stokes, 1953).

Just as glucose was the only aldose to act as an adequate substrate for growth, so was it the only aldose to be extensively oxidized by washed organisms. The first stages of the oxidation of glucose to gluconate and thence to 2- and 5-oxogluconate are considered unlikely to involve phosphorylation of the substrates. Thus the formation of the keto acids by washed organisms was insensitive to high concentrations of fluoride and to a concentration of phenylmercury acetate which abolished the phosphorylation of glucose by the soluble fraction of ultrasonic extracts (Fewster, 1957*b*); the rates of aerobic oxidation of glucose and gluconate by a particulate fraction rich in cytochrome of such extracts were not dependent upon the addition of ATP and the two keto acids were the only products detected (Fewster, 1957*a*). Similar observations have been made concerning the oxidation of glucose and gluconate to 2-oxogluconate by *Pseudomonas aeruginosa* (Stokes & Campbell, 1951) and *Pseudomonas fluorescens* (Wood & Schwerdt, 1953).

D-Arabonate, formed from 2-oxogluconate by the action of washed organisms, is considered to be an end product; the existence of an alternative pathway for 2-oxogluconate breakdown is indicated by the demonstration of the presence of a 2-oxogluconokinase in ultrasonic extracts (Fewster, 1957*b*). In addition, evidence has been presented by Kovachevich & Wood (1955) for the operation of the Entner–Doudoroff pathway in *A. suboxydans*. There is a requirement for the presence of any one of a number of readily oxidized substrates for the initiation of the oxidation of 5-oxogluconate (with small amounts of added substrates their oxidation was complete long before that of 5-oxogluconate ceased). This suggests that energy is required for the uptake of the keto acid by the organisms. Hauge, King & Cheldelin (1955) showed that pre-incubation of washed suspensions of *A. suboxydans* with a readily oxidized carbohydrate resulted in the organisms acquiring the ability to oxidize dihydroxyacetone rapidly. Tosic (1946) was able to reduce the lag period of acetate oxidation by suspensions of *Acetobacter turbidans* by the addition of small amounts of a variety of compounds, and suggested that these compounds caused the activation of one of the enzymes essential for acetate oxidation. The problem of the pathway of oxidation of 5-oxogluconate is worthy of attention.

With the exception of glucose 6-phosphate, D-glucurone and L-rhamnose all aldoses tested were oxidized, at various rates, to an extent corresponding to the formation of aldonic acids; only 2-deoxy-, 3-*O*-methyl- and 6-*O*-methyl-D-glucose and D-mannose were further oxidized, with negligible evolution of carbon dioxide, to give reducing acids. The non-oxidation of glucose 6-phosphate may be explained in terms of impermeability, since it has been shown to be oxidized by ultrasonic extracts (Fewster, 1957*a*). The presence of a complete pentose cycle in cell-free extracts of *A. suboxydans* has been demonstrated by Hauge *et al.* (1955); the non-utilization of the various aldoses other than glucose beyond preliminary stages indicates the absence of phosphorylating or isomerizing mechanisms or both enabling these compounds to enter the main metabolic pathway.

The specificity of the oxidative enzyme system of the cytochrome-deficient organisms resulting from growth in media containing glucose in the absence of chalk resembles that of the flavoprotein-glucose oxidase or notatin of *Penicillium notatum* (Keilin & Hartree, 1948*a*, 1952); this latter enzyme has recently been shown by Sols & de la Fuente (1957) to oxidize 2-deoxy-D-glucose, although at only 25% the rate of the oxidation of glucose. Notatin can also utilize phenolindo-2:6-dichlorophenol as a hydrogen acceptor (Keilin & Hartree, 1948*a*). However, no evidence was obtained for the formation of hydrogen peroxide in the present study and, unlike notatin, the rate of oxidation of glucose was sensitive to cyanide under aerobic conditions and triphenyltetrazolium chloride did not serve as a hydrogen acceptor under anaerobic conditions. The properties of the enzyme also differed from those of a cyanide-sensitive oxidase found in a solubilized particulate fraction of *A. suboxydans* by King & Cheldelin (1957), which was shown to be cytochrome-dependent and to oxidize galactose as well as glucose.

SUMMARY

1. The effectiveness of various carbohydrates as energy sources for the growth of *Acetobacter suboxydans* in complex and semi-defined media is described; maximum growth was obtained with D-mannitol and D-sorbitol, good growth with glycerol, D-glucose in the presence of chalk, and D-fructose, and poor growth with calcium D-gluconate; negligible growth resulted with sodium D-gluconate, L-sorbose, D-mannose, D-galactose, D-xylose, L-arabinose and sucrose.

2. The extent of oxidation of glucose by washed organisms was influenced by the age and pH of the culture and the energy source for growth, and was sensitive to cyanide, fluoride, 2:4-dinitrophenol and phenylmercury acetate.

3. Maximum oxidation of glucose by washed organisms occurred with an uptake of 3·75 moles of oxygen and an output of up to 4·0 moles of carbon dioxide/mole of glucose; gluconate and 2- and 5-oxogluconate were shown to be intermediates and are considered to be formed without the participation of phosphate esters.

4. By the action of washed organisms 2-oxogluconate underwent oxidation and decarboxylation to give arabonate; 5-oxogluconate, in the presence of added readily oxidized substrate, was extensively oxidized with an uptake of oxygen of up to 3·5 moles and an output of carbon dioxide of 4·0 moles/mole.

5. Glucose was the only aldose to be extensively oxidized by washed organisms; some derivatives of glucose, other hexoses, pentoses and aldehydes were oxidized to a limited extent. A number of intermediates of the tricarboxylic acid cycle and acids formed during the growth of *Acetobacter* and *Pseudomonas* species in media containing glucose were not oxidized.

6. Organisms grown on a medium containing glucose and under conditions where acid production was allowed to proceed unchecked were shown to lack the characteristic cytochrome spectrum of *A. suboxydans* and to oxidize only glucose and 2-deoxy-D-glucose to the corresponding aldonic acids.

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The Isolation of 3β-Hydroxy-Δ⁵-Steroids from the Urine of Normal Men

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Dehydro*epi*androsterone (3β-hydroxyandrost-5-en-17-one) was the first steroid containing the 5-en-3β-ol group to be isolated from urine (Butenandt & Dannenbaum, 1934). During the succeeding twenty-two years only one other steroid of this type, namely androst-5-ene-3β:16α:17β-triol (Marrian & Butler, 1944), was isolated from the urine of normal subjects, although a number of steroids of this type were isolated from the urine of patients with an adrenal cortical tumour. More recently, Fotherby, Colas, Atherden & Marrian (1957) obtained from extracts of the urine of normal males a previously undescribed substance which was characterized as 16α-hydroxydehydro*epi*androsterone (3β:16α-dihydroxyandrost-5-en-17-one). This compound was liberated by boiling the urine without adjustment of the pH, a procedure which appears to be specific for the hydrolysis of the 3β-sulphates of 3β-hydroxy-Δ⁵-steroids (cf. Roy, 1956). By use of this method the extracts obtained were less contaminated with non-steroid material or with other steroids than were extracts of urine hydrolysed in other ways and the transformations which the 3β-hydroxy-Δ⁵-steroids undergo during acid hydrolysis were avoided. By analogy with dehydro*epi*androsterone, which appears to be present in urine as the 3β-sulphate and 16α-hydroxydehydro*epi*androsterone, it seemed likely that other 3β-hydroxy-Δ⁵-steroids (if present in urine) would also be conjugated with sulphuric acid and should therefore be present in extracts of boiled urine. Accordingly, a search for 3β-hydroxy-Δ⁵-steroids in the urine of normal males was initiated.

RESULTS

Isolation of crystalline compounds from urine extracts

In the present work a large volume of urine from normal males was hydrolysed by boiling for 2 hr. without adjustment of pH. It would appear from the work of Dingemanse, Huis in't Veld & Hartogh-Katz (1952), Teich, Rogers, Lieberman, Engel & Davis (1953) and Fotherby *et al.* (1957) that the major reaction product resulting from boiling a solution of the sulphate of a 3β-hydroxy-Δ⁵-steroid at neutral pH is the 3:5-*cyclo*-6β-hydroxy steroid accompanied by a small amount of the 3β-hydroxy-Δ⁵-steroid. For this reason the residue obtained by evaporation of chloroform extracts of boiled urine was dissolved in aqueous ethanol and the solution acidified to pH 1 in order to convert the 3:5-*cyclo*-6β-hydroxy steroids into 3β-hydroxy-Δ⁵-steroids. The concentrate thus obtained was separated into a non-ketonic fraction and a ketonic fraction by means of the Girard reaction carried out at room temperature and each fraction subjected to countercurrent distribution.

Examination of the non-ketonic fraction

This fraction was first distributed for 200 cycles by using the solvent system ethyl acetate–*cyclo*hexane–ethanol–water (2:8:3:7, by vol.) described by Engel, Alexander, Carter, Elliott & Webster (1954). The modified Pettenkofer reaction described by Munson, Jones, McCall & Gallagher (1948), which has been shown to be fairly specific for 3β-hydroxy-Δ⁵-steroids, has been used throughout the examination of the non-ketonic fraction to follow the degree of resolution obtained during the countercurrent distributions and chromatographic separations. This preliminary distribution separated the non-ketonic fraction into a less 'polar' fraction containing those substances with a partition coefficient greater than one and a more 'polar' fraction containing those substances with a partition coefficient less than one. The less 'polar' fraction after distribution in a different solvent system for 300 cycles was almost completely separated into two components. The faster-moving component was crystallized from methanol and shown to be identical with androst-5-ene-3β:17β-diol. The slower-moving component, also after crystallization from methanol, melted at 213–217°, which suggested that the compound might be pregn-5-ene-3β:17α:20α-triol; this suggestion was proved to be correct by comparison of the acetate of the isolated compound with authentic 3β:20α-diacetoxypregn-5-en-17α-ol.

Countercurrent distribution of the more 'polar' non-ketonic fraction for 100 cycles with the solvent system *cyclo*hexane–ethyl acetate–ethanol–water (80:20:45:45, by vol.) gave a distribution pattern which suggested the presence of at least two components. By recycling the upper phases for a

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further 400 transfers, these two components were completely separated and the slower-moving component shown to be androst-5-ene-3β:16α:17β-triol. After chromatography of the faster-moving component on a magnesium silicate–Celite column to remove interfering pigments, and crystallization from methanol, 9 mg. of material was obtained which melted at 259–262° and did not depress the melting point of authentic androst-5-ene-3β:16β:-17β-triol. A mixture of the isolated compound and androst-5-ene-3β:16α:17β-triol melted at 235–240°. The melting point of the acetate of the isolated compound was not depressed by admixture with authentic 3β:16β:17β-triacetoxyandrost-5-ene and the spectra of solutions of the acetates in concentrated sulphuric acid were identical.

Examination of the ketonic fraction

This was subjected to countercurrent distribution for 180 cycles in the solvent system ethyl acetate–*cyclo*hexane–ethanol–water (2:8:3:7, by vol.) and alternate fractions were analysed for 3β-hydroxy-Δ⁵-steroids by the modified Pettenkofer reaction, for oxo steroids by the Zimmermann reaction and for α-ketolic compounds by the 'blue tetrazolium' reaction. The distribution pattern showed the presence of two peaks giving a positive Pettenkofer reaction. The faster-moving of these was shown to be dehydro*epi*androsterone. The slower-moving peak was a mixture of at least two compounds: one reacted positively in the blue-tetrazolium reaction and negatively in the Zimmermann reaction and had a partition coefficient of 0·52; the second component reacted negatively in the blue-tetrazolium reaction and positively in the Zimmermann reaction and had a partition coefficient of 0·65. By partition chromatography on a Celite column the blue-tetrazolium-reactive material was further separated from the material giving a positive Zimmermann reaction and shown to be identical with 16α-hydroxydehydro*epi*androsterone. Owing to lack of material the compound reacting positively in the Zimmermann test has not yet been identified. However, if the compound is a steroid it is possible that it has not previously been isolated from urine, as its melting point (219–220°) does not correspond with that of any 3β-hydroxy-Δ⁵-steroid so far isolated from natural sources.

EXPERIMENTAL

Methods

Modified Pettenkofer reaction. This was performed as described by Munson *et al.* (1948), except that only 0·2 ml. of acetic acid, 0·8 ml. of furfuraldehyde soln. and 3 ml. of H_2SO_4 were used.

Blue-tetrazolium reaction. The reaction described by Mader & Buck (1952) was slightly modified. To the dry residue to be analysed were added 0·25 ml. of 1% (w/v) tetramethylammonium hydroxide in 95% ethanol and 0·35 ml. of 0·07% (w/v) blue tetrazolium [3:3′-dianisolebis-4:4′-(3:5-diphenyl)tetrazolium chloride] in 95% ethanol. After incubation for 1 hr. at 25° in the dark the mixture was diluted with 4 ml. of 1% (v/v) acetic acid in 50% ethanol and the optical density of the soln. measured at 510 mμ.

Zimmermann reaction. A scaled-down version of the reaction described by Callow, Callow & Emmens (1938) has been used. To the material to be analysed 0·24 ml. of a mixture of 2 vol. of 1% (w/v) *m*-dinitrobenzene in ethanol and 1 vol. of 2·5% (w/v) potassium hydroxide in ethanol were added. After 1 hr. at 25° the soln. was diluted with 4 ml. of ethanol and the optical densities were measured in a Unicam spectrophotometer SP. 600 at 440, 490, 520, 540 and 600 mμ. The corrected optical density at 520 mμ was calculated from the readings at 440, 520 and 600 mμ as recommended by Allen (1950), and indicated the presence of 17-oxo steroids. Similarly, the corrected optical density at 490 mμ (the wavelength at which 20-oxo steroids show maximum absorption) was calculated from the readings at 440, 490 and 540 mμ.

Spectra of solutions of compounds in concentrated sulphuric acid. The method described by Zaffaroni (1950) has been used. Between 70 and 100 μg. of steroid was dissolved in 4 ml. of conc. H_2SO_4 and after the soln. had stood for 2 hr. at 25° the optical density over the wavelength range 220–600 mμ was determined. The figures recorded below in the sections describing the isolation of steroids from urine show the wavelengths of the maxima and minima in the absorption spectra, the $E^{1\%}_{1\,cm.}$ value at these wavelengths being given in parentheses.

Miscellaneous. Melting points were determined on a microscope hot-stage and are uncorrected for emergent stem.

Samples for C and H analysis and for optical-rotation measurements were dried to constant weight at 80° *in vacuo* over P_2O_5.

The solvents and Celite (Celite 535, Johns-Manville Co. Ltd., London) used were purified by the methods employed by Watson & Marrian (1956). Magnesium trisilicate (British Drug Houses Ltd.) was activated by drying overnight at 110°. Celite columns for partition chromatography were prepared as described by Bauld (1955).

For the countercurrent distributions a 100-tube all-glass fully automatic apparatus of the type described by Craig, Hausmann, Ahrens & Harfenist (1951) was used. Upper- and lower-phase volumes each of 40 ml. have been used throughout the investigation.

Preparation of urine extracts for countercurrent distribution

Urine from normal men was collected in batches of 20 l. without preservative and concentrated *in vacuo* to approx. 2 l. The concentrated urine was boiled for 2 hr. without adjustment of pH and extracted twice with an equal vol. of $CHCl_3$. The combined $CHCl_3$ extracts were washed twice with 0·2 vol. of 0·1 N-NaOH and twice with 0·2 vol. of water, dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The residue thus obtained was dissolved in 28 ml. of methanol, the soln. diluted with 12 ml. of water and extracted once with 40 ml. of hexane. The hexane extract was back-extracted with 40 ml. of 70% (v/v) aqueous methanol and the combined aqueous

methanol phases were evaporated under reduced pressure to about 20 ml. After dilution to about 60 ml. with water the aqueous solution was extracted twice with 100 ml. of ether, the combined ether extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness.

The residues were stored in the dry state at $-20°$ until 250 l. of urine had been processed and the combined residues then separated into a non-ketonic fraction and a ketonic fraction by use of Girard's reagent T according to the procedure described by Fotherby *et al.* (1957). Each fraction was then dissolved in 200 ml. of a water–ethanol (9:1, v/v) mixture and acidified to pH 1 with 5N-H_2SO_4 and after 1 hr. the mixture was extracted twice with 2 vol. of freshly distilled ether. The ether extracts were washed twice with 0·2 vol. of 0·1N-NaOH and twice with 0·2 vol. of water, dried over Na_2SO_4 and evaporated to dryness under reduced pressure. The residues representing the ketonic and non-ketonic fractions were stored at $-20°$ in the dry state until 2000 l. of urine had been processed.

Examination of the non-ketonic fraction

The dry residue of the non-ketonic fraction (2·5 g.) was distributed by means of the solvent system *cyclo*hexane–ethyl acetate–ethanol–water (8:2:3:7, by vol.). After completion of the fundamental procedure 100 fractions were collected by single withdrawal. Because of a mechanical fault it was necessary to combine the material in tubes 0–90 of the apparatus (the more 'polar' fraction) and to combine the material in tubes 91–99 with those fractions collected by single withdrawal (the less 'polar' fraction). Analysis of tubes 0–100 of the apparatus before combination of the contents showed that by the above division no compound giving a positive Pettenkofer reaction would appear in both fractions.

Less 'polar' non-ketonic fraction. This fraction was placed in the first two tubes of the countercurrent apparatus and distributed by means of the solvent system *cyclo*hexane–ethyl acetate–ethanol–water (10:90:45:55, by vol.). After completion of the fundamental procedure tubes 38 to 99 were emptied to dispose of the main bulk of pigmented impurities. The tubes were then refilled with lower phase, the fundamental procedure again completed and the upper phases recycled for a further 200 cycles. Two peaks of material reacting positively in the Pettenkofer reaction were detected, one occupying tubes 19–40 and the second occupying tubes 41–91.

Isolation of androst-5-ene-3β:17β-diol. The contents of tubes 41–91 were combined and crystallized three times from methanol to give 50 mg. of white crystals, which had m.p. 170–173°. The mixed m.p. with an authentic sample of androst-5-ene-3β:17β-diol (m.p. 176–179°) was 175–178°. A portion of the isolated compound (15 mg.) was acetylated with acetic anhydride and pyridine at room temperature overnight; the acetate, after two crystallizations from methanol, had m.p. 153–157°, unchanged by admixture with an authentic sample of 3β:17β-diacetoxyandrost-5-ene (Found C, 73·9; H, 9·4. Calc. for $C_{23}H_{34}O_4$: C, 73·8; H, 9·2%). The spectra of solutions of the isolated and authentic compounds in conc. H_2SO_4 were identical [max. 310 mμ ($E^{1\%}_{1\,cm.}$ 145), 350 mμ (135), 410 mμ (280); min. 260 mμ (50), 332 mμ (128), 370 mμ (118); inflexion 480 mμ (115)].

Isolation of pregn-5-ene-3β:17α:20α-triol. The contents of tubes 19–40 were combined and crystallized three times from methanol, to give 70 mg. of crystals which had m.p. 213–217°; $[\alpha]_D^{16} - 82°$ in ethanol (*c*, 0·488) (Found: C, 75·3; H, 10·3. Calc. for $C_{21}H_{34}O_3$: C, 75·4; H, 10·2%). On drying a sample of this compound at 80° *in vacuo* for 3 hr. it lost 2·2% of its weight but regained it rapidly (within 10 min.) on exposure to the atmosphere (see also Hirschmann & Hirschmann, 1950*b*). The compound formed an acetate which had m.p. 198–200°, unchanged by admixture with an authentic sample of 3β:20α-diacetoxypregn-5-en-17α-ol, and the spectra of solutions of the acetates of the isolated and authentic compounds in conc. H_2SO_4 were identical [max. 344 mμ ($E^{1\%}_{1\,cm.}$ 180), 412 mμ (300), 442 mμ (250), 500 mμ (220); min. 260 mμ (71), 370 mμ (130), 430 mμ (245), 471 mμ (198)].

More 'polar' non-ketonic fraction. This fraction was placed in the first two tubes of the countercurrent apparatus and distributed by means of the solvent system *cyclo*hexane–ethyl acetate–ethanol–water (80:20:45:55, by vol.). After completion of the fundamental procedure the upper phases were recycled for 400 transfers. The distribution pattern showed two peaks of material reacting positively in the Pettenkofer reaction, one occupying tubes 0–32 and the second occupying tubes 33–80.

Isolation of androst-5-ene-3β:16β:17β-triol. The contents of tubes 33–80 were combined and the deeply pigmented residue obtained was chromatographed on a column (28 cm. × 2 cm.) containing 60 g. of a mixture of magnesium trisilicate and Celite (2:1 by wt.) prepared in light petroleum (boiling range 80–100°). Elution was commenced with 10 ml. of light petroleum from a reservoir containing 400 ml. of light petroleum and continued with a mixture of light petroleum and ethanol, the ethanol concentration being increased in a stepwise discontinuous gradient by adding 1 ml. of ethanol to the reservoir for every 10 ml. of liquid removed from it. Material reacting positively in the Pettenkofer reaction appeared in the eluate when 350 ml. of eluent had passed through the column and was then completely eluted from the column with 200 ml. of 5% ethanol in light petroleum and 50 ml. of 20% ethanol in light petroleum. The material was crystallized twice from methanol, to give 9 mg. of crystals which had m.p. 259–262° unchanged by admixture with authentic androst-5-ene-3β:16β:17β-triol. A mixture of the isolated compound and androst-5-ene-3β:16α:17β-triol had m.p. 235–240°. The compound formed an acetate which after crystallization from methanol had m.p. 206–209° unchanged by admixture with authentic 3β:16β:17β-triacetoxyandrost-5-ene (m.p. 208–211°) (Found: C, 69·3; H, 8·3. Calc. for $C_{25}H_{36}O_6$: C, 69·4; H, 8·3). The compound had $[\alpha]_D^{18} - 35°$ in ethanol (*c*, 0·2) and the spectra of solutions of the isolated and authentic acetates in conc. H_2SO_4 were identical [max. 410 mμ ($E^{1\%}_{1\,cm.}$ 280), 475 mμ (83); min. 265 mμ (37), 450 mμ (70); inflexion 350 mμ (120)].

Isolation of androst-5-ene-3β:16α:17β-triol. The contents of tubes 0–32 were combined and crystallization from methanol–benzene yielded 11 mg. of crystals which had m.p. 258–262° unchanged by admixture with authentic androst-5-ene-3β:16α:17β-triol. It formed an acetate which after crystallization from methanol had m.p. 178–181° unchanged by admixture with authentic 3β:16α:17β-triacetoxyandrost-5-ene. The spectra of solutions of the isolated and authentic acetates in conc. H_2SO_4 were identical [max. 360 mμ ($E^{1\%}_{1\,cm.}$ 136), 410 mμ (342), 470 mμ (166); min. 245 mμ (30), 368 mμ (133), 443 mμ (142)].

Examination of the ketonic fraction

The dry residue of the ketonic fraction (1·6 g.) was distributed by means of the solvent system *cyclo*hexane–ethyl acetate–ethanol–water (8:2:3:7, by vol.). After completion of the fundamental procedure 80 fractions were collected by single withdrawal and at the end of the distribution alternate fractions were analysed by the Pettenkofer, Zimmermann and blue-tetrazolium reactions. The distribution pattern showed two peaks of material reacting positively in the Pettenkofer reaction.

Isolation of dehydroepiandrosterone. The material comprising one peak (partition coefficient 30) also reacted positively in the Zimmermann reaction and after crystallization from methanol had m.p. 135–137°, 147–149°, unchanged by admixture with authentic dehydro*epi*androsterone.

Isolation of 16α-*hydroxydehydroepiandrosterone.* The second peak of material reacting positively in the Pettenkofer reaction extended from tube 43 to tube 90. Analyses by the Zimmermann and blue-tetrazolium reactions showed the peak to consist of two components. One of these, which occupied tubes 43–81 (partition coefficient 0·52), reduced blue tetrazolium but did not react positively in the Zimmermann reaction. The second component, which occupied tubes 52–90 (partition coefficient 0·65), reacted positively in the Zimmermann reaction but did not reduce blue tetrazolium. The contents of tubes 43–90 were combined and chromatographed on a column (80 cm. × 2 cm.) of 120 g. of Celite with the solvent system benzene–hexane–methanol–water (7:3:8:2, by vol.). Successive 25 ml. fractions were collected and analysed. The Zimmermann-reactive material appeared in fractions 26–42 and the blue-tetrazolium-reducing material in fractions 35–48. Fractions 40–48 were combined and chromatographed on a column (28 cm. × 2 cm.) of 60 g. of Celite with the solvent system methanol–water (80%, v/v)–benzene. The blue-tetrazolium-reducing material, eluted by 200–320 ml. of solvent, was free of the Zimmermann-reactive material, and after two crystallizations from methanol it had m.p. 175–179°, unchanged by admixture with authentic 16α-hydroxydehydro*epi*androsterone.

DISCUSSION

Since the work of Ronzoni (1952), Dingemanse *et al.* (1952) and Cohen & Oneson (1953) dehydro*epi*androsterone has been recognized as one of the major constituents of the 17-oxo steroid fraction of the urine of normal people. However, the possibility that other 3β-hydroxy-Δ⁵-steroids might be present in the urine of normal people has not previously been systematically investigated. Thus, of the compounds whose isolation is reported here, androst-5-ene-3β:17β-diol and pregn-5-ene-3β:17α:20α-triol have not previously been isolated from this source, and the isolation of androst-5-ene-3β:16β:17β-triol from natural sources is reported for the first time. Large amounts of 3β-hydroxy-Δ⁵-steroids have been found in the urine of subjects with an adrenal-cortical tumour by numerous investigators, the most thorough studies being those of Hirschmann & Hirschmann (1945, 1947, 1950*a*, *b*) and Hirschmann (1943), who isolated from the urine of a boy with this condition seven 3β-hydroxy-Δ⁵-steroids, namely dehydro*epi*androsterone, androst-5-ene-3β:17β-diol, androst-5-ene-3β:16α:17β-triol, pregn-5-ene-3β:20α-diol, pregn-5-ene-3β:16α:20α-triol, 3β:17α-dihydroxypregn-5-en-20-one and pregn-5-ene-3β:17α:20α-triol. Of these, pregn-5-ene-3β:20α-diol, pregn-5-ene-3β:16α:20α-triol and 3β:17α-dihydroxypregn-5-en-20-one have not been isolated in the present work.

The isolation of androst-5-ene-3β:17β-diol and androst-5-ene-3β:16β:17β-triol is of interest in that it extends the analogy between the metabolism of dehydro*epi*androsterone and that of oestrone [3-hydroxyoestra-1:3:5(10)-trien-17-one]. Hirschmann (1943) and Marrian & Butler (1944) have suggested that androst-5-ene-3β:16α:17β-triol arises from dehydro*epi*androsterone by a metabolic route similar to that by which oestriol [oestra-1:3:5(10)-triene-3:16α:17β-triol] is formed from oestrone. The conversion of dehydro*epi*androsterone into androst-5-ene-3β:16α:17β-triol has been demonstrated both in man (Mason & Kepler, 1947) and with rabbit-liver slices (Schneider & Mason, 1948). Fotherby *et al.* (1957) have suggested that 16α-hydroxydehydro*epi*androsterone may be an intermediate in this transformation and have shown that 16α-hydroxydehydro*epi*androsterone is a metabolic product of dehydro*epi*androsterone in man. This metabolic pathway is similar to that suggested by Marrian, Watson & Panattoni (1957*b*) and Marrian, Loke, Watson & Panattoni (1957*a*) for the conversion of oestrone into oestriol via 16α-hydroxyoestrone [3:16α-dihydroxyoestra-1:3:5(10)-trien-17-one], which they have isolated from the urine of pregnant women. Marrian *et al.* (1957*b*) have further suggested that 16β-hydroxyoestrone (3:16β-dihydroxyoestra-1:3:5(10)-trien-17-one) might be the intermediate in the conversion of oestrone into 16-*epi*oestriol [oestra-1:3:5(10)-triene-3:16β:17β-triol], which has been isolated from pregnancy urine by Marrian & Bauld (1955). Similarly, the androst-5-ene-3β:16β:17β-triol isolated in the present work might arise from dehydro*epi*androsterone via 16β-hydroxydehydro*epi*androsterone.

The interconvertibility of oestrone and oestradiol-17β [oestra-1:3:5(10)-triene-3:17β-diol] in humans is well known, and the analogous reactions between dehydro*epi*androsterone and androst-5-ene-3β:17β-diol have also been demonstrated. Thus Mason & Kepler (1945) have isolated androst-5-ene-3β:17β-diol from the urine of a man with pituitary insufficiency who was receiving dehydro*epi*androsterone, and Ungar, Miller & Dorfman (1954) have isolated dehydro*epi*androsterone from the urine of a man to whom androst-5-ene-3β:17β-diol had been

administered. There is, however, one difference between the metabolism of oestrone and that of dehydro*epi*androsterone. The conversion of oestrone into the other oestrogens discussed above has been assumed to be the major metabolic pathway for oestrone, whereas it is known that for dehydro*epi*androsterone the major metabolic route is conversion into 3α-hydroxy-5α-androstan-17-one and 3α-hydroxy-5β-androstan-17-one and metabolism to compounds retaining the 5-en-3β-ol group is a minor metabolic pathway.

While it is thus possible to fit the C_{19} 3β-hydroxy-Δ^5-steroids isolated in the present work into a metabolic scheme the position of pregn-5-ene-3β:17α:20α-triol is at present obscure. It could be the precursor of the urinary dehydro*epi*androsterone, and Lieberman & Teich (1953) have suggested that this compound is a metabolite of an intermediate in the biosynthesis of the adrenal hormones.

SUMMARY

1. Androst-5-ene-3β:17β:-diol, androst-5-ene-3β:16β:17β-triol and pregn-5-ene-3β:17α:20α-triol have been isolated from normal male urine which has been boiled for 2 hr. at neutral pH.

2. By analogy with dehydro*epi*androsterone and 16α-hydroxydehydro*epi*androsterone it is likely that these steroids are present in urine as the 3β-sulphates.

3. The metabolism of dehydro*epi*androsterone is compared with that of oestrone.

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The Biochemistry of Aromatic Amines

5. 2-ACETAMIDO-6-HYDROXY-5-NAPHTHYL HYDROGEN SULPHATE AND OTHER METABOLITES OF 2-NAPHTHYLAMINE AND 2-ACETAMIDONAPHTHALENE*

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Exposure to 2-naphthylamine is known to cause cancer of the bladder, but not of any other organ in man. For this and other reasons the carcinogenic effect is thought to be exerted through some metabolite excreted in the urine. The metabolism of this and other aromatic amines has therefore been intensively studied, and a list of the known metabolites of 2-naphthylamine is given in Table 1. 2-Acetamido-6-hydroxy-5-naphthyl hydrogen sulphate (described in the present paper) has been isolated as a metabolite of 2-acetamidonaphthalene and detected as a metabolite of 2-naphthylamine.

EXPERIMENTAL

Methods

Animals. Animals were kept in metabolism cages and the urine was collected daily. Rabbits (average wt. 2 kg.) were fed on cabbage, bran and water, and rats on rat cake, bread and water. Rabbits were injected intraperitoneally with 0·5 g. of 2-naphthylamine or 0·5 g. of 2-acetamidonaphthalene in 10 ml. of arachis oil. Rats (average wt. 200 g.) were dosed in the same way with 0·05 g. of either compound in 1 ml. of oil.

Paper chromatography. Whatman no. 1 paper was employed with the following solvent systems: (*a*) butanol–propan-1-ol–aq. 0·1 N-NH_3 soln. (2:1:1, by vol.), (*b*) butanol–acetic acid–water (2:1:1, by vol.) and (*c*) butanol–acetic acid–water (70:5:19, by vol.) and Whatman no. 3 MM paper was used with the solvent system (*d*) butanol–acetic acid–water (10:1:1, by vol.).

Reagents used in this work and R_F values of some 2-naphthylamine derivatives have been described previously (Booth, Boyland & Manson, 1955). Acetamido compounds and hydrogen sulphates were hydrolysed on chromatograms by spraying the paper with N-HCl and heating between glass sheets for 0·5 hr. at 70° in an oven. A Hanovia Chromatolite was used as a source of ultraviolet light. 2-Amino-1-naphthol hydrochloride could not be run on chromatograms without oxidation to give coloured compounds at the solvent front and along the line of flow. The use of a solvent system containing HCl (Ekman, 1948) did not prevent this. If the paper was pretreated by spraying with ascorbic acid (1% in water) until just moist and then dried with gentle heat, 2-amino-1-naphthol hydrochloride could then be run in solvent system (*b*)

* Part 4: Boyland & Manson (1957).

Table 1. *Metabolites of 2-naphthylamine*

References: 1, Wiley, 1938; 2, Dobriner *et al.* 1941; 3, Boyland & Manson, 1957; 4, Boyland & Manson, present work; 5, Boyland *et al.* 1957; 6, Manson & Young, 1950.

Compound	Reference to Identification	Reference to Isolation
2-Acetamidonaphthalene	—	2, 4
2-Naphthylsulphamic acid	—	5
2-Naphthylamine *N*-glucosiduronic acid	—	5
2-Amino-1-naphthyl hydrogen sulphate	—	1, 5, 6
2-Amino-1-naphthyl glucosiduronic acid	3	—
2-Amino-1-naphthyl hydrogen sulphate *N*-glucosiduronic acid	5	—
2-Amino-6-naphthol	2, 4	—
2-Amino-6-naphthyl hydrogen sulphate	4	—
2-Amino-6-naphthyl glucosiduronic acid	3	—
2-Acetamido-6-naphthol	—	2, 6, 4
2-Acetamido-6-naphthyl hydrogen sulphate	4	—
2-Acetamido-6-naphthyl glucosiduronic acid	—	3
2-Acetamido-6-hydroxy-5-naphthyl hydrogen sulphate	—	4
2-Acetamido-5:6-dihydro-5:6-dihydroxynaphthalene	4	—
2-Acetamido-5:6-dihydro-5:6-dihydroxynaphthalene glucosiduronic acid	3	—

(R_F 0·85) without much oxidation (cf. paper chromatography of adrenaline and noradrenaline, Crawford & Outschoorn, 1951). The aminonaphthol could then be detected by Ehrlich's reagent or by the characteristic bluish-purple colour on exposure to NH_3 vapour. Aqueous solutions of 2-amino-1-naphthol hydrochloride give a green colour on the addition of aq. NH_3 soln. or other alkali. If the solution is then shaken with benzene, the organic phase becomes purple (Liebermann & Jacobson, 1882).

Urine was applied directly to the chromatograms or concentrated before application by adsorption on charcoal deactivated with stearic acid (7·5% in ethanol) (Asatoor & Dalgliesh, 1956). Urine (10 ml.) was passed under suction through a charcoal column (2·0 cm. × 3·5 cm.) and the column was then washed with water (3 × 25 ml.) and aq. phenol soln. (5%, 100 ml.). The phenol eluate was evaporated to dryness under reduced pressure. Residual phenol was removed in a desiccator over NaOH.

Materials

All analytical samples were dried at 0·5 mm. Hg and 100° for 2 hr. Most of the compounds used have been described previously (Booth *et al.* 1955). 2-Diacetamidonaphthalene was prepared by the method of Sudborough (1901).

2-Acetamido-5:6-diacetoxynaphthalene. 6-Acetamido-1:2-naphthaquinone (Kehrmann & Matis, 1898) was reduced to the corresponding dihydroxy compound by the method of Fieser & Fieser (1939) for the reduction of 1:2-naphthaquinone. The finely powdered quinone (0·7 g.) was added in portions over 15 min. to $Na_2S_2O_4$ (1·4 g.) in water (20 ml.) with shaking and slight warming on the water bath. The quinone decolorized rapidly and the product remained mainly in suspension. After cooling, the buff-coloured solid (0·6 g.) was collected, washed with water and dried. The product was not purified, but it gave one spot (R_F 0·9) on an ascending chromatogram developed in solvent system (*b*). The spot was faintly fluorescent, became greenish brown on exposure to light and reddish blue on spraying with dilute Na_2CO_3 soln. but gave no additional colour with diazotized sulphanilic acid. After hydrolysis on the chromatogram the spot gave an orange colour with Ehrlich's reagent. The compound could not be run in solvent system (*a*) without the formation of coloured products. The dihydroxy compound was kept overnight in pyridine (2 ml.) and acetic anhydride (2 ml.) at room temperature. The solution was poured into water and the precipitate collected and crystallized from aq. ethanol to yield *2-acetamido-5:6-diacetoxynaphthalene* (plates), m.p. 172–174° (Found: C, 64·1; H, 5·1; N, 4·4. $C_{16}H_{15}O_5N$ requires C, 63·8; H, 5·0; N, 4·65%).

Potassium 2-acetamido-6-hydroxy-5-naphthyl sulphate. 6-Nitro-2-naphthylamine was prepared from 2-naphthylamine (Saunders & Hamilton, 1932) and converted into 2-nitro-6-naphthol (Veselý & Jakes, 1923). A saturated aq. solution of $K_2S_2O_8$ (7 g.) was added with stirring to 2-nitro-6-naphthol (5 g.) and KOH (5 g.) in water (100 ml.) over 4 hr. After being kept overnight at room temperature, the solution was adjusted to pH 5·0 and extracted several times with ether. The aqueous phase was made alkaline again with KOH and evaporated to dryness under reduced pressure. The residue was crystallized from aq. ethanol to give *dipotassium 2-nitro-6-hydroxy-5-naphthyl sulphate* (1·6 g.), as red needles blackening at 200° but not melting below 360° (Found: C, 31·5; H, 2·1; N, 3·7; S, 8·1; K, 20·4. $C_{10}H_5O_7NSK_2,H_2O$ requires C, 31·65; H, 1·9; N, 3·5; S, 8·4; K, 20·6%). The nitro compound (0·7 g.) was shaken with hydrogen and Adam's catalyst in aq. ethanol. After filtration the solution was evaporated to dryness under reduced pressure. The solution darkened considerably. The residue was acetylated with thioacetic acid (0·4 g.) in pyridine (10 ml.) (cf. Booth *et al.* 1955). After being kept at room temperature overnight, the solution was evaporated to dryness in a desiccator over P_2O_5 and NaOH. The residue was crystallized from aq. ethanol to give *potassium-2-acetamido-6-hydroxy-5-naphthyl sulphate*, m.p. 185–188° (decomp.) (Found: C, 41·0; H, 3·4; N, 3·9; S, 8·6; K, 10·8. $C_{12}H_{10}O_6NSK,H_2O$ requires C, 40·8; H, 3·4; N, 4·0; S, 9·1; K, 11·1%). On paper chromatograms the compound had R_F values of 0·45 (descending) and 0·5 (ascending) in solvent system (*a*) and 0·57 (ascending) in solvent system (*b*). The spot became greenish brown upon exposure to daylight. It gave a pale-pink colour on spraying with Na_2CO_3 and diazotized sulphanilic acid solutions, but did not diazotize and couple with hexylresorcinol. After acid treatment of the chromatogram at 70° the spot diazotized (greenish-yellow colour), but on spraying with alkaline hexylresorcinol it gave the same dirty red colour as it gave on spraying with alkali alone without diazotization. After hydrolysis on the chromatogram the spot gave an orange colour with Ehrlich's reagent. After hydrolysis in solution it gave a red colour on addition of alkali, changing to dirty green with shaking. On extraction with benzene, the organic phase was purple but faded after 30 min. The last colour reaction resembles that of 2-amino-1-naphthol except that with this compound the benzene has a darker red tinge and is stable for several hours. Potassium 2-acetamido-6-hydroxy-5-naphthyl sulphate was not hydrolysed by the sulphatase of Taka-diastase (Parke, Davis and Co. Ltd.).

The triacetyl derivative of 2-amino-5:6-dihydroxynaphthalene was prepared as follows. The nitro compound (0·6 g.) was reduced as before and the solution evaporated to dryness under reduced pressure. The residue was dissolved in water and acidified with conc. HCl to precipitate 2-amino-6-hydroxy-5-naphthyl hydrogen sulphate. This compound was not purified. It gave a mauve colour in alkaline solution with diazotized sulphanilic acid and a bluish-red colour on diazotization and coupling with hexylresorcinol. It had R_F values of 0·32 (ascending), with a green-coloured tail, in solvent system (*a*) and 0·5 (ascending), with tailing, in solvent system (*b*). The compound was hydrolysed by heating with 5N-HCl at 100° for 30 min. The solution was cooled and the 2-amino-5:6-dihydroxynaphthalene collected, and acetylated with acetic anhydride in pyridine. The product was crystallized from aq. ethanol to yield plates, m.p. 173–174°, which did not depress the melting point of 2-acetamido-5:6-diacetoxynaphthalene prepared from the reduction of 6-acetamido-1:2-naphthaquinone. Attempts to prepare 2-acetamido-6-hydroxy-5-naphthyl hydrogen sulphate by treating 2-acetamido-6-naphthol with alkaline persulphate were unsuccessful and none was detected in the reaction mixture by paper chromatography.

RESULTS

Isolation of metabolites

2-Naphthylamine, 2-acetamidonaphthalene and 2-acetamido-6-naphthol. 2-Naphthylamine (0·5 g. in 10 ml. of arachis oil) was injected intraperitoneally into each of five

rabbits for 11 days (total 27·5 g.) and the urine collected daily. The urine was adjusted to pH 6·5 and continuously extracted with ether for 16 hr. The ether extract was washed several times with 2N-HCl. The washings were made alkaline and the precipitate was collected and crystallized from aq. ethanol to give 2-naphthylamine (0·6 g., 2% of the dose), m.p. and mixed m.p. 109–111° (Found: N, 9·6. Calc. for $C_{10}H_9N$: N, 9·8%). The ether extracts were evaporated to small bulk and cooled to give crude 2-acetamido-6-naphthol (3·5 g.), m.p. 190–199°. Two crystallizations from aq. ethanol gave 1 g. (equivalent to 2% of the dose) of the acetamidonaphthol, m.p. and mixed m.p. 220–222° (Found: C, 71·9; H, 5·8; N, 6·95. Calc. for $C_{12}H_{11}O_2N$: C, 71·6; H, 5·5; N, 6·9%). The ethereal solution was diluted with more ether and washed with 2N-NaOH to remove residual 2-acetamido-6-naphthol, followed by water, and then dried and evaporated to dryness. The residue was extracted several times with hot light petroleum (b.p. 80–100°) and the combined extracts were evaporated to dryness. Crystallization of the residue from aq. ethanol gave 2-acetamidonaphthalene (3 mg.), m.p. and mixed m.p. 129–131°. After rabbits had been injected with 2-acetamidonaphthalene, 0·2 g. of 2-acetamido-6-naphthol was isolated from a total dose of 16·8 and 0·8 g. from a total dose of 11·5 g. These yields were equivalent to 1 and 6% of the administered material. Dobriner, Hoffman & Rhoads (1941) isolated these compounds, in unstated yields, from rats, rabbits and monkeys dosed with 2-naphthylamine. Manson & Young (1950) isolated 2-acetamido-6-naphthol from rats injected with 2-naphthylamine and 2-acetamidonaphthalene, and recovered 2-naphthylamine and 2-acetamidonaphthalene from the urine of rats injected with these compounds.

2-Acetamido-6-hydroxy-5-naphthyl hydrogen sulphate. In the isolation of 2-acetamido-6-naphthyl glucosiduronic acid from rabbits injected with 28 g. of 2-acetamidonaphthalene a new compound was encountered on separating the metabolites on a cellulose column (Boyland & Manson, 1957, p. 277). From its behaviour in solvent system (*a*) it appeared to be a hydrogen sulphate. The fraction eluted from the cellulose column contained both this and 2-acetamido-6-naphthyl hydrogen sulphate. The eluate was made alkaline with aq. KOH soln. and evaporated to 5 ml. After cooling at 5° for 5 days, crystals separated which were collected and recrystallized from aq. ethanol to yield plates (0·09 g.) of potassium 2-acetamido-6-hydroxy-5-naphthyl sulphate, m.p. and mixed m.p. 188–189° (decomp.) (Found: C, 40·8; H, 3·5; N, 4·2; S, 9·0; K, 11·0. Calc. for $C_{12}H_{10}O_6NSK,H_2O$: C, 40·8; H, 3·4; N, 4·0; S, 9·1; K, 11·1%). The infrared spectrum was identical with that of the synthetic ester. The compound gave a precipitate on heating with $BaCl_2$ in 2N-HCl. The colour reactions and R_F values were identical with those of the synthetic compound and it showed the same resistance to hydrolysis by Taka-diastase. The compound was not easily detected in untreated urine by one-dimensional paper chromatography in solvent system (*a*) as the spot was almost in the same position as indoxyl hydrogen sulphate and 2-acetamido-6-naphthyl hydrogen sulphate. It was more readily detected after adsorption of the metabolites on charcoal followed by elution with phenol. The residue from the eluate was examined by two-dimensional paper chromatography in solvent system (*a*) followed by (*b*). The spot was then detected by the colour formed on exposure to light for several hours. The metabolite was also detected in the urine of rabbits and rats dosed with 2-naphthylamine. In solvent system (*d*) the compound had the same R_F (0·4, descending) as 2-acetamido-6-naphthyl hydrogen sulphate and indoxyl hydrogen sulphate. The mother-liquors from the isolation of the metabolite contained 2-acetamido-6-naphthyl hydrogen sulphate, identified by its colour reactions and by the release of 2-acetamido-6-naphthol on incubation with Taka-diastase.

Paper chromatography of urine after injection of 2-naphthylamine and 2-acetamidonaphthalene

Rats and rabbits were injected with 2-naphthylamine and the urine was examined for other metabolites than those already described by Boyland, Manson & Orr (1957) and Boyland & Manson (1957). 2-Naphthylamine, 2-amino-1-naphthyl hydrogen sulphate and 2-acetamido-6-naphthol were identified in the urine of both species. 2-Amino-6-naphthol and its hydrogen sulphate were found in rat urine, although not consistently, but never in rabbit urine. For the identification of 2-acetamidonaphthalene solvent system (*c*) was used. In this system 2-naphthylamine had an R_F value of 0·90 and the acetamido derivative one of 0·95. In the urine of both species a non-fluorescent spot was found which agreed with the R_F value of 2-acetamidonaphthalene and which diazotized and coupled only after acid treatment of the chromatograms. 2-Diacetamidonaphthalene, however, behaved in the same way. The hydrogen sulphates of 2-amino-1-naphthol and of 2-amino-6-naphthol and their corresponding acetamido derivatives were separated by descending development in solvent system (*d*) on no. 3MM paper (for R_F values see Booth *et al.* 1955). By development in this solvent system and acid treatment of the chromatogram at 70°, 2-acetamido-6-naphthyl hydrogen sulphate was identified in the urine of both species but 2-acetamido-1-naphthyl hydrogen sulphate was not found in either species. The detection of 2-amino-1-naphthol after hydrolysis of the synthetic hydrogen sulphate was carried out by exposing the spot to ammonia vapour, when a characteristic blue colour was obtained.

Both rat and rabbit urine contained a compound believed to be 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene. The occurrence of this compound as a glucosiduronic acid and some reasons for the structure suggested have been described (Boyland & Manson, 1957). The unconjugated compound was not readily extracted by ether even by continuous extraction but was extracted by ethyl acetate or butanol. The compound was not detected in the urine of rabbits dosed with 2-amino-6-naphthol, only 2-amino-6-naphthol and 2-acetamido-6-naphthol and their hydrogen sulphates and glucosiduronic acids being found.

Neither 2-amino-1-naphthol nor 2-acetamido-1-naphthol was detected in the urine of rats or rabbits by paper chromatography. The injection of 2-acetamidonaphthalene into rabbits gave the same metabolites in the urine as injection of 2-naphthylamine, except that no 2-naphthylamine was detected. The injection of 2-acetamidonaphthalene into rats gave the same compound as injection of the parent amine except that 2-amino-6-naphthol and its hydrogen sulphate were not found.

Chemical oxidation of 2-acetamidonaphthalene

2-Acetamidonaphthalene (0·9 g.) was oxidized by the chemical hydroxylating system (ascorbic acid, ferrous sulphate, ethylenediaminetetra-acetic acid and oxygen) of Udenfriend, Clark, Axelrod & Brodie (1954) and Brodie, Axelrod, Shore & Udenfriend (1954), as employed by Booth *et al.* (1955). The reaction mixture was extracted several times with ether and then with ethyl acetate. The ethereal extracts contained the compounds giving the reactions and R_F values [0·95 and 0·92 in solvent system (*a*)] of 2-acetamido-1-naphthol and 2-acetamido-6-napthol (Booth *et al.* 1955). The ethyl acetate extracts were evaporated to dryness and examined by paper chromatography. They contained a compound [R_F 0·8 in solvent system (*a*)] with identical properties to those of the metabolite described in this paper and by Boyland & Manson (1957), which is believed to be 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene. A two-dimensional ascending chromatogram was carried out, developing first in solvent system (*a*), then treating with acid at 70°, drying and developing in the second direction with solvent system (*b*). A spot [R_F 0·8 in solvent system (*b*)] with the colour reactions of 2-amino-6-naphthol was detected.

DISCUSSION

2-Acetamido-6-hydroxy-5-naphthyl hydrogen sulphate is a new metabolite of 2-naphthylamine and 2-acetamidonaphthalene. Boyland & Sims (1957) isolated an analogous compound, 2-hydroxy-1-naphthyl hydrogen sulphate, from rabbits dosed with naphthalene. Other workers (Corner & Young, 1954, 1955; Sato, Yamada, Suzuki, Fukuyama & Yoshikawa, 1956; Solomon, 1954) detected this conjugate in the urines of several species after the injection of naphthalene. Dobriner *et al.* (1941) stated that they had isolated an unknown metabolite from the urine of rats injected with 2-naphthylamine and that its properties suggested that it was a dihydroxyaminonaphthalene but it was not further characterized. The urine of rabbits and monkeys contained only traces of this compound.

Evidence for the presence of 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene is confined to the behaviour of the suspected metabolite on paper chromatograms (this paper and Boyland & Manson, 1957), but the presence of this compound would be expected by analogy with the metabolism of naphthalene (Young, 1947; Booth & Boyland, 1949). Moreover, in the metabolism of naphthalene, 1:2-dihydro-1:2-dihydroxynapthalene appears to be an intermediate in the formation of 1:2-dihydroxynaphthalene (Corner & Young, 1955; Sato *et al.* 1956). 2-Naphthylamine and 2-acetamidonaphthalene are metabolized to similar derivatives. Of the metabolites detected by paper chromatography, 2-amino-1-naphthyl hydrogen sulphate has been isolated from the urine of various species after administration of 2-naphthylamine (Boyland *et al.* 1957; Manson & Young, 1950; Wiley, 1938) and 2-acetamido-6-naphthol has been isolated from the urine of animals injected with 2-naphthylamine and 2-acetamidonaphthalene (Dobriner *et al.* 1941; Manson & Young, 1950). The latter may be an artifact formed by enzymic hydrolysis of conjugates in the urine. 2-Acetamidonaphthalene is also a known metabolite of 2-naphthylamine (Dobriner *et al.* 1941). The identical properties of 2-diacetamidonaphthalene and 2-acetamidonaphthalene in the solvent systems used do not allow the former compound to be excluded as a metabolite. Peters & Gutmann (1955) found that 2-diacetamidofluorene was a metabolite of 2-aminofluorene in rat-liver slices.

2-Acetamido-1-naphthyl hydrogen sulphate was not detected in the urine of either species. Booth *et al.* (1955) found that this compound was deacetylated by rat-kidney and -liver slices and also that 2-amino-1-naphthyl hydrogen sulphate was not acetylated by these tissues. Hence the excretion of 2-acetamido-1-naphthyl hydrogen sulphate is unlikely and in fact only 2-amino-1-naphthyl hydrogen sulphate was detected. On the other hand, 2-acetamido-6-naphthyl hydrogen sulphate was not deacetylated by rat-liver and -kidney slices and 2-amino-6-naphthyl hydrogen sulphate was acetylated. 2-Acetamido-6-naphthyl hydrogen sulphate was detected in the urine of both species. The metabolites of 2-naphthylamine are listed in Table 1.

SUMMARY

1. The isolation of 2-acetamido-6-hydroxy-5-naphthyl hydrogen sulphate from the urine of rabbits injected with 2-acetamidonaphthalene is described. The compound was detected in the urine of rabbits and rats injected with 2-naphthylamine.

2. The compound was synthesized by the persulphate oxidation of 2-nitro-6-naphthol followed by reduction and acetylation with thioacetic acid.

3. The urine of rats and rabbits injected with 2-naphthylamine was examined by paper chromatography. 2-Naphthylamine, 2-acetamidonaphthalene, 2-acetamido-6-naphthol, 2-amino-1-naphthyl hydrogen sulphate, 2-acetamido-6-naphthyl hydrogen sulphate and a compound which is probably 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene were detected. Rat urine sometimes contained 2-amino-6-naphthol and its hydrogen sulphate. The injection of 2-acetamidonaphthalene gave the same products except that 2-naphthylamine was not detected in rabbit urine and 2-amino-6-naphthol and its hydrogen sulphate were not detected in rat urine. 2-Amino-1-naphthol, 2-acetamido-1-naphthol and 2-acetamido-1-naphthyl hydrogen sulphate were not detected as metabolites of 2-naphthylamine or 2-acetamidonaphthalene in the urine of rats or rabbits.

4. The oxidation of 2-acetamidonaphthalene by an ascorbic acid–ferrous sulphate–oxygen system yields a compound which is probably 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene.

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The Amino Acid Composition of Cytochrome *c* from Horse Heart

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Though the physiological properties of haemoproteins such as haemoglobin, catalase, peroxidase and cytochrome *c* ultimately depend on the structure of their common prosthetic group they differ so widely that the properties of the prosthetic group must be greatly affected by the nature of the protein to which it is attached and the way in which it is bound to the protein. Our understanding of this problem is limited largely by our ignorance of the structure of the proteins involved. A certain amount is known about the relation between the protein and haem parts of the molecule of cytochrome *c*. Theorell (1938, 1939) discovered that the prosthetic group is firmly attached to the protein by thioether linkages with two cysteine residues. Tsou (1951) subsequently isolated from a peptic hydrolysate of cytochrome *c* a peptide carrying the prosthetic group, and the amino acid sequence of this peptide has been worked out (Tuppy & Paléus, 1955; Leaf & Gillies, 1955). Tuppy & Bodo (1954) also examined similar peptides obtained by tryptic and acid hydrolysis. As none of these peptides possesses the specific enzymic properties of cytochrome *c*, however, the remainder of the peptide chain must contribute significantly to the properties of the intact protein.

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It seems necessary therefore to know the structure of the whole protein moiety before the problem of its association with the prosthetic group can be solved.

The first step in the investigation of the structure of a protein is usually the determination of its amino acid composition. Though Keilin & Hartree (1937) analysed cytochrome *c* for basic amino acids and cystine, it subsequently transpired that their preparation was impure and the first extensive study of what would now be regarded as a pure sample of the protein was made by Theorell & Åkeson (1941). Their success was limited by the methods of amino acid analysis then available. Since then methods have greatly improved and it appeared desirable to investigate the problem with the elegant methods of amino acid analysis developed by Moore and Stein (Stein & Moore, 1948; Moore & Stein, 1949, 1951). Since this work was undertaken three other amino acid analyses of cytochrome *c* have been published, one of cytochrome *c* from cow heart by Theorell & Ehrenberg (1955) and two of horse-heart cytochrome *c* by de Barbieri & Zamboni (1951) and de Barbieri & Scevola (1955). These are discussed below.

EXPERIMENTAL

Materials

Cytochrome c. This was prepared from fresh horse heart by the method of Keilin & Hartree (1945). Care was taken to prevent frothing, particularly when the protein was in acid solution, as this appeared to promote denaturation. The final dialysis was carried out against distilled water rather than sodium chloride solution and the solution then lyophilized. The product varied greatly in purity, from run to run, containing from 0·30 to 0·42% of Fe. It was further purified by chromatography on the ion-exchange resin Amberlite IRC-50. This method was first described by Paléus & Neilands (1950) and was later studied by Margoliash (1954). The conditions used in this Laboratory differed from those used by these workers in that sodium phosphate buffers were used to develop the columns. The finely divided resin (100–240 mesh) was purified by washing with acid and alkali and stored dry in the hydrogen form. Columns (3 cm. × 20 cm.) were prepared by a method similar to that described by Hirs, Moore & Stein (1953), being poured in phosphate buffer (pH 7·0, 0·162 M-Na^+). Phosphate buffer was allowed to pass through the column until the effluent and influent solutions had the same pH and optical density at 280 mμ. The cytochrome *c* (200–500 mg.) was dissolved in 2–5 ml. of phosphate buffer (0·1 M, pH 7·0). This solution was run into the top of the column and the chromatogram developed with the same buffer. A light-brown band ran rapidly down the column. This material showed a weak metmyoglobin spectrum and was probably a mixture of metmyoglobin and its globin as suggested by Margoliash (1954). Two further bands moved more slowly down the column, a narrow pink band consisting of the ferrous form of cytochrome *c*, and following it the main band of the ferric cytochrome. Near the top of the column there remained a number of narrow reddish-brown bands which probably consisted of cytochrome *c* modified by the action of trichloroacetic acid. The elution with 0·1 M-buffer was continued until the main band was approximately half way down the column, by which time the optical density of the effluent had returned to its initial value, and the concentration of the buffer was then increased to 0·25 M. This accelerated the movement of the main band and yielded the purified cytochrome in more concentrated solution. This procedure gave a more efficient separation of the oxidized and reduced forms of the cytochrome than the method of Margoliash (1954), and eliminated the necessity of repeating the fractionation at pH 9. The main fraction of the cytochrome moved down the column as a symmetrical band which appeared to be homogeneous by spectrophotometric analysis of different fractions of the eluate at 550 and 280 mμ. The eluate containing the oxidized cytochrome was dialysed against distilled water until free of phosphate, and either lyophilized or stored at −20°.

The activity of the cytochrome *c* in the succinoxidase system (Potter & Dubois, 1942) bore a constant relation to its light absorption at 550 mμ during this stage of its purification, and the spectral properties of the final product were not affected by carbon monoxide (Tsou, 1951). It was assumed therefore that the protein had not undergone denaturation. The analytical values for the purified protein are shown in Table 1, where they are compared with those obtained by other workers. The homogeneity of the preparation was tested in the Tiselius apparatus and gave a single peak both in phosphate buffer (pH 7·0, *I* 0·2) and veronal buffer (pH 8·5, *I* 0·2). Analysis of samples from different parts of the U-tube after electrophoresis yielded the same analytical figures as the starting material.

Table 1. *Analytical values of cytochrome* c *preparations*

	Present work	Margoliash (1955) Horse heart	Tint & Reiss (1950)		Paléus & Neilands (1950) Cow heart	Theorell & Åkeson (1941)
Iron (%)	0·460	0·465	0·456	0·453	0·466	0·43
$10^{-4}\,\epsilon_{550\,m\mu}$ (reduced)	2·76	2·77	2·78	2·64	2·69	2·81
$\frac{E_{550\,m\mu}\text{ (reduced)}}{E_{280\,m\mu}\text{ (oxidized)}}$	1·20	1·19	—	—	1·17	—
Nitrogen (%)	15·98	—	—	—	—	15·10
Mol. wt.	12 100	12 000	12 270	12 350	11 980	13 000

Reagents. The reagents used were as far as possible of analytical-reagent quality. The amino acids used for recovery experiments were obtained from British Drug Houses Ltd. and their purity was checked by determination of carbon, hydrogen and nitrogen content and by paper chromatography. We are indebted to Mr J. L. Cameron of the Chemistry Department for the microanalyses.

Methods

Amino acid analysis. The protein was hydrolysed by heating in a sealed tube with 100 times its weight of glass-distilled 6N-HCl at 110° for 24 hr. The HCl was then removed by repeated evaporation *in vacuo* over NaOH and H_2SO_4. The residue was dissolved and made up to a known volume with the solvent which was to be used for chromatographic analysis and samples were taken for determination of total nitrogen. No attempt was made to determine 'humin nitrogen', though a small quantity of humin was present. The hydrolysate was qualitatively analysed by paper chromatography with butanol–acetic acid–water (4:1:5, by vol.; Partridge, 1948) and saturated aq. phenol soln. in the presence of ammonia (Dent, 1948). The dried chromatograms were sprayed with a 0·2% (w/v) solution of ninhydrin in aq. 50% (v/v) ethanol containing 1% (by vol.) of both acetic acid and collidine.

The quantitative analyses were at first carried out on starch columns as described by Stein & Moore (1948) and Moore & Stein (1949), with potato starch obtained from Gordon Slater Co. The results obtained were as described by these authors except that the separation of alanine and glutamic acid with 2-methylpropan-2-ol–butan-2-ol–0·1N-HCl (2:1:1) was unsatisfactory. This separation was later achieved by chromatography on ion-exchange resin (Moore & Stein, 1951). The resin used was Zeo-Karb 225 with a nominal degree of cross-linking of 8% and average particle size of 50 μ. In this case the resolution was entirely satisfactory. No attempt was made to determine the basic amino acids on the 100 cm. columns, which were stopped after the emergence of phenylalanine. The basic amino acids and ammonia were determined separately with 15 cm. columns, which also yielded a combined value for phenylalanine and tyrosine.

The effluent from the columns was collected in fractions of 1 ml. and the amino acid content of these was determined with the ninhydrin reagent of Moore & Stein (1948). The reagent (1 ml.) was used for each analysis with a heating time of 30 min. and the optical density was measured in the Unicam SP. 500 or SP. 600 spectrophotometer. Standard curves (optical density/concentration) were prepared for each amino acid and the relative colour values agreed with those reported by Moore & Stein (1948). In our experience the ninhydrin reagent did not always keep for more than a week and it was found desirable to analyse a standard amino acid solution with each batch of column fractions. The standard solution was prepared by dilution of a stock solution of glycine (AnalaR) to 0·4 mM with the same solvent as was present in the column fractions to be analysed.

Determination of cyst(e)ine by oxidation to cysteic acid. Preliminary experiments suggested that bromine water was a milder reagent for this purpose than performic acid and yet gave a quantitative yield of cysteic acid when acting on standard solutions of cystine. The amino acid solution or protein hydrolysate was made N with respect to HCl and a saturated solution of bromine in water was added dropwise until the solution had a persistent but faint yellow colour. The solution was then evaporated in a desiccator over NaOH and H_2SO_4, dissolved in citrate buffer (pH 3·42) and run through a 100 cm. column of Zeo-Karb 225 prepared as for the other amino acid analyses. The cysteic acid emerged after 25 ml. of the buffer had passed through the column and was therefore well separated from all other amino acids present.

RESULTS

The results of the analyses are presented in Table 2. The results of qualitative analysis are implicit in the list of amino acids given. Serine was absent from all pure preparations and indeed its presence in a hydrolysate was taken as a sign of impurity in the sample of cytochrome used. In addition to the amino acids listed in Table 2 tryptophan was present. This amino acid was not detected by paper chromatography as only acid hydrolysates were examined by this technique, but it was shown to be present by the method of Graham, Smith & Hier (1947) with the intact protein. In Table 2 the values obtained by the use of starch and ion-exchange columns are presented separately as these techniques provide a differential analysis insofar as the amino acids emerge from the respective columns in different orders. It might therefore be expected that fortuitous contamination of particular amino acid fractions would not be the same for both types of column. The close agreement between the two sets of results therefore lends them greater significance.

The numbers in parentheses after the analytical values in Table 2 represent the occasions on which the particular amino acid was sufficiently separated from others for individual determination. On starch chromatograms developed with butanol–propanol–0·1N-HCl, resolution is incomplete and the following groups of amino acids are found in mixed fractions: leucine and isoleucine; valine, tyrosine and methionine; alanine and glutamic acid. The first two groups were separated on chromatograms developed with butanol–benzyl alcohol–water and the means of results of two such analyses are given in Table 2. These results agree with and are therefore supported by combined values obtained on four other columns where resolution of these particular amino acids was incomplete. The same applies to the results for phenylalanine and tyrosine obtained from ion-exchange chromatograms. These amino acids were determined separately on 100 cm. columns on two occasions and as a combined fraction on the short columns used primarily for the separation of the basic amino acids on four other occasions. As mentioned in the Experimental section alanine and glutamic acid were not separated on starch chromatograms, but

for ease of presentation the combined value for these amino acids obtained from starch chromatograms has been divided in proportion to the individual values obtained from the ion-exchange chromatograms. This makes it possible to correct for the loss of glutamic acid due to esterification on the starch columns.

The mean total recoveries of nitrogen from the starch columns was 94·3 % and from the ion-exchange columns 94·8 %. If allowance is made for the prosthetic group and tryptophan (assuming one residue of tryptophan/molecule of cytochrome *c*) these values are raised to 101·35 and 101·65 respectively. The mean-weight recoveries (based on residue weights), for the three analyses for which it was determined, was 90·5 %, which with allowance for one residue of tryptophan and the prosthetic group is raised to 97·6 %. The weight recovery was determined only in some experiments as it was thought that the weighing of the small amounts of protein used for the analyses was less accurate than duplicate determinations of nitrogen.

Recoveries of individual amino acids were determined for both starch and ion-exchange resin chromatograms before analysis of cytochrome *c* hydrolysates, but when values for the composition of the cytochrome *c* hydrolysate became available an amino acid mixture made up to have the same composition was analysed by ion-exchange chromatography. The percentage recoveries of the amino acids from this mixture are shown in Table 2. In general the recoveries were similar to those obtained by Moore & Stein (1951) but those of cystine, methionine, isoleucine and arginine were low.

The poor recovery of the sulphur-containing amino acids was presumably caused by their oxidation during passage through the column. The presence of freshly distilled thiodiglycol in the eluting buffer and the use of boiled buffer did not appreciably improve the situation. The value of 62 % for the recovery of cystine was based on three determinations for which the individual values were 50, 67 and 69 %. The degree of loss is therefore sufficiently consistent to allow of correction of the values obtained for the cyst(e)ine content of the hydrolysate if it is assumed that the loss is the same for the experimental as for the control chromatograms. A suitable correction has therefore been applied to the value given in Table 2. No value for cyst(e)ine was obtained from the starch chromatograms, on which this amino acid was completely destroyed. The figure quoted in Table 2 cannot therefore be regarded as very reliable. An attempt was made to overcome this difficulty by oxidizing the cystine to cysteic acid before chromatography of the hydrolysate. In an artificial mixture of amino acids cystine was oxidized quantitatively to cysteic acid and the recovery of this acid from ion-exchange columns was complete. When, however, a sample of the protein hydrolysate was treated in

Table 2. *Amino acid composition of cytochrome* c

The number of separate estimations on which each value is based is given in parentheses.

	Starch columns		Ion-exchange columns		
	Amino acid N as % of total N	Residues/ mole of cytochrome *c*	Amino acid N as % of total N	Residues/ mole of cytochrome *c*	Recovery from amino acid mixture (%)
Glycine	7·72 (4)	10·6	7·94 (2)	10·9	99
Alanine	3·92*	5·4	3·77 (2)	5·2	97
Valine	1·60 (2)	2·2	1·53 (2)	2·1	98
Leucine	3·94 (2)	5·4	3·81 (2)	5·2	97
Isoleucine	3·97 (2)	5·4	3·70 (2)	5·1	92
Proline	2·60 (4)	3·5	2·10 (1)	2·9	97
Phenylalanine	2·25 (5)	3·1	2·34 (2)	3·2	104
Tyrosine	2·61 (2)	3·6	2·75 (2)	3·8	97
Threonine	6·24 (4)†	8·6	6·37 (2)†	8·8	100
Cyst(e)ine	Lost	—	1·66 (2)‡	2·2	62
Methionine	0·98 (2)	1·3	Lost	—	40
Arginine	5·51 (5)	1·9	5·19 (4)	1·8	91
Histidine	4·44 (4)	2·0	5·64 (5)	2·6	98
Lysine	26·75 (5)	18·3	25·75 (5)	17·8	95
Aspartic acid	5·18 (5)	7·1	5·15 (2)	7·1	98
Glutamic acid	8·00*	11·0	7·92 (2)‡	10·8	94
Ammonia	5·08 (5)	7·0	6·24 (3)	8·5	100

* Alanine and glutamic acid were not separated on starch columns. The mean combined value from five determinations has been divided in proportion to the separate values obtained for these amino acids from ion-exchange chromatograms. Allowance has been made for 7% loss of glutamic acid on starch columns owing to esterification.

† Corrected for 8 % loss on hydrolysis.

‡ Corrected for loss on column.

the same way the yield of cysteic acid corresponded to 1·38 residues of cysteine/molecule of cytochrome *c*, similar to the yield without oxidation. This suggests that some constituent of the hydrolysate, perhaps a breakdown product of the prosthetic group, interferes with the oxidation of cystine. Schram, Moore & Bigwood (1954) recommend the oxidation of the cyst(e)ine residues in the intact protein, but it is doubtful whether this method could be applied successfully to cytochrome *c* as the effect of the oxidizing agent on the thioether linkages between the protein and the prosthetic group cannot be predicted.

In contrast with cystine, methionine could be determined on starch but not on Zeo-Karb 225. When the artificial mixture of amino acids was analysed on an ion-exchange column some 60 % of the methionine was lost, but from the cytochrome hydrolysate the loss of methionine was practically complete. Fortunately the conditions used for the determination of this amino acid on starch columns were less destructive. From an amino acid mixture 98 % of the added methionine was recovered. The value obtained for the methionine content of the cytochrome hydrolysate is not, however, as precise as this would suggest. The amount of hydrolysate which can be separated on a starch column with butanol–benzyl alcohol–water as eluting solvent is severely limited by the disturbing effect of the hydrophilic amino acids, which are present in unusually high proportion in cytochrome *c*, on the equilibrium between the starch and the solvent. This meant that the methionine peak was low and the accuracy of the determination reduced. It is thought that the value obtained is low rather than high and therefore indicates the presence of two residues of methionine in the protein molecule.

The low recovery of isoleucine was at first puzzling, as the sample of the amino acid used in making up the amino acid mixture gave satisfactory analytical figures for carbon, hydrogen and nitrogen. The explanation may, however, be found in the observations of Piez (1954) and van Dam-Bakker (1958) that commercial samples of synthetic isoleucine usually contain some *allo*isoleucine which runs with methionine on the ion-exchange column. The low recovery of methionine makes it impossible to determine from our results whether this is the true explanation but it suggests that correction of the isoleucine values obtained by analysis of the hydrolysate is unnecessary.

The low value for the recovery of arginine from the ion-exchange resin column may be due to error in the ninhydrin determination rather than incomplete elution from the column. Arginine is eluted from the column as a rather broad band and as there is only a small quantity present this means that the ninhydrin-colour values given by the tubes containing arginine are low. Any uncertainty in the blank value can therefore quite easily introduce an error of 10 % into the determination. In spite of this uncertainty the analyses indicate fairly clearly that there are two residues of arginine in the molecule of cytochrome *c*.

The recovery of other amino acids and ammonia lay between 95 and 100 % for both starch and ion-exchange chromatograms. The values obtained by the two methods agree well with one another except for those of ammonia, histidine and proline. The value for ammonia obtained from the ion-exchange chromatogram is appreciably greater than that obtained on starch chromatograms but it is thought that the lower value is more reliable. The ion-exchange resin may pick up traces of ammonia inadvertently present in the buffers used for the preparation of the column and this ammonia will subsequently be eluted from the column at about the same time as the ammonia added in the hydrolysate. This is not likely to happen with a starch column. Of the two values obtained for histidine, that obtained from ion-exchange columns is taken to be the more accurate. The precision of the determination of histidine on starch columns is limited by the same factors as were mentioned in connexion with the determination of arginine. The low colour value given by proline in the ninhydrin reaction similarly reduces the accuracy with which this amino acid can be determined. It is impossible on the basis of the present analyses to say whether there are three or four residues of proline in the molecule of cytochrome *c*.

Loss of amino acids during the process of hydrolysis. The process of hydrolysis itself causes loss of some amino acids. The values for threonine have been corrected for loss with the factor found by Hirs, Stein & Moore (1954) for similar conditions of hydrolysis. This correction is arbitrary, particularly in view of the findings of Smith & Stockell (1954) and Smith, Stockell & Kimmel (1954), but even Smith's method of determining the loss involves the assumption that the rate of loss is constant throughout the period of hydrolysis. Tryptophan, as was expected, appeared to be destroyed completely during hydrolysis. An attempt was made to determine the tryptophan content of the intact protein by the method of Graham *et al.* (1947). The result obtained corresponded to 0·58 residue/molecule of cytochrome, which suggests that the method does not work very well for this protein, but in the absence of better results may be taken to show the presence of one residue of tryptophan in the cytochrome molecule. Direct spectrophotometric methods such as that of Holiday & Ogston (1938) cannot be used owing to interference by the prosthetic group.

DISCUSSION

The results expressed as residues/mole of cytochrome *c* corrected to the nearest whole number are presented in Table 3. For comparison the values obtained by de Barbieri & Scevola (1955) for cytochrome *c* prepared from horse heart and cow heart and those of Theorell & Ehrenberg (1955) for cytochrome *c* prepared from cow heart are also included. The earlier results of de Barbieri & Zamboni (1951) differ so widely from those given in Table 3 that they have not been included. The values of de Barbieri & Scevola (1955), quoted in Table 3, show closer agreement with results of the present work on the one hand and those of Theorell & Ehrenberg (1955) on the other. There are, however, marked discrepancies, and it is thought that the results of de Barbieri & Scevola (1955), which were obtained by paper chromatography, are more likely to be in error than results obtained by column chromatography. The technique of quantitative paper chromatography requires further refinement before it is adequate for purposes of this sort. There is in general closer agreement between our results and those of Theorell & Ehrenberg (1955) for cow-heart cytochrome *c*. It is not clear from the report of the Swedish workers whether cow-heart cytochrome *c* contains serine. If it does not, the most significant difference between the two proteins lies in their content of glycine. Another notable difference is that most of the values for cow-heart cytochrome are rather higher than ours for the protein from horse heart. It must be noted, however, that calculation of the molecular weight from the figures of Theorell & Ehrenberg (1955) gives a value of some 15 600. This is much higher than their value of 13 000, determined in the ultracentrifuge, and the value of approximately 12 000 calculated from the iron content of the purest preparations (Paléus & Neilands, 1950). This may have a bearing on the calculation of specific volume of the protein made by Theorell & Ehrenberg. They obtained a value of 0·737 without considering the prosthetic group. As the observed value for the specific volume of cytochrome *c* is 0·702–0·707 they concluded that the prosthetic group must take up very little space. The value calculated from our results (Table 3) is 0·708, which makes any such assumption about the packing of the prosthetic group unnecessary.

In view of the observations of Theorell & Åkeson (1941) not much difference would be expected between the two proteins in respect of the balance of acidic and basic groups, and this is borne out by the figures in Table 3. The number of histidine residues present is of particular interest, as Theorell has suggested that two of these residues are bound to the iron atom of the prosthetic group. Margoliash (1955), by means of paper chromatography, obtained a value of four residues of histidine/mole of horse-heart cytochrome *c*. From our own results it seems very unlikely that there are more than three. If the value for ammonia in Table 3 is taken as a measure of the number of

Table 3. *Comparison of the amino acid compositions of cytochrome* c *from heart of horse and cow*

Values are given in terms of residues of amino acid/molecule of cytochrome *c*. The values from the present work are corrected to the nearest whole number (cf. Table 2), those of Theorell & Ehrenberg (1955) are quoted as they were published and those of de Barbieri & Scevola (1955) are calculated from the percentages given by these authors, assuming a molecular weight of 12 000.

	Present work	de Barbieri & Scevola (1955)	Theorell & Ehrenberg (1955)	
	Horse heart		Cow heart	
Glycine	11	8·6	8·8	15·5
Alanine	5	5·3	7·0	6·6
Valine	2	3·5	2·8	3·3
Leucine	5	7·5	7·9	6·2
Isoleucine	5			6·2
Proline	3	4·3	6·0	3·9
Phenylalanine	3	2·8	3·3	3·3
Tyrosine	4	2·1	2·3	3·8
Tryptophan	1	—	—	1·0
Serine	0	0	1·2	8·5
Threonine	9	7·8	5·7	
Cyst(e)ine	2	1·0	2·7	2·0
Methionine	2	1·1	1·1	2·0
Arginine	2	3·2	3·0	3·4
Histidine	3	3·1	5·2	3·1
Lysine	18	12·9	12·2	18·3
Aspartic acid	7	7·8	9·6	9·4
Glutamic acid	11	8·6	8·3	11·9
Ammonia	7	—	—	—

amide groups in the molecule there is an excess of some 12 basic groups over acidic groups, and this is in keeping with the basic properties of cytochrome *c*.

The values quoted for the sulphur-containing amino acids in Table 3 require comment. Our experience suggests that the methods used in the present work are not suitable for accurate determination of these amino acids, but in the absence of other values we have put forward those that appear most likely, that is, two methionine and two cysteine residues/molecule of cytochrome *c*. It is not known whether the conditions of hydrolysis used here were sufficiently vigorous to split the thioether bonds linking the prosthetic group to cysteine residues in the protein. If so, these may be the only cysteine residues in the protein. Åkeson (1942) and Paléus (1955) concluded that this was true of cytochrome *c* from cow heart. Carruthers (1947), on the other hand, obtained evidence that even in this protein there are cysteine residues other than those involved in the binding of the prosthetic group. It may be that Carruthers's polarographic data are open to other interpretations but further examination of this problem seems desirable.

SUMMARY

1. Cytochrome *c* was prepared from horse heart and purified by chromatography on Ambèrlite IRC-50. The product contained 0·46 % of iron and 15·98 % of nitrogen.

2. The amino acid composition of the protein was determined by means of starch and ion-exchange chromatography.

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The Assimilation of Amino Acids by Bacteria

25. THE PREPARATION AND ACTIVITIES OF A FACTOR INVOLVED IN THE INCORPORATION OF AMINO ACIDS IN DISRUPTED STAPHYLOCOCCAL CELLS*

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In previous papers of this series (Gale & Folkes, 1955*a*, *b*) a description has been given of the preparation of disrupted cells of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) which retain the ability to synthesize proteins and to incorporate labelled amino acids into the protein of the preparation. Incubation of the preparation with ribonuclease or deoxyribonuclease, or extraction with M-sodium chloride, results in a loss of these abilities. The synthesis of proteins can be restored by addition to the incubation mixture of suitable combinations of nucleic acids or purines and

* Part 24: Gale & Folkes (1957).

pyrimidines, the particular combinations required depending upon the degree of depletion of nucleic acid in the preparation and on the protein or enzyme whose synthesis is being studied (Gale & Folkes, 1955*b*). The incorporation of amino acids is restored by the addition of staphylococcal nucleic acid to the incubation mixture; deoxyribonucleic acid (DNA) is more effective than ribonucleic acid (RNA) when compared on a basis of optical density and restores incorporation to a level higher than that attained in the presence of RNA (Gale & Folkes, 1955*a*). The action of RNA in restoring the incorporation of glycine is not abolished by digestion with ribonuclease, the activity of the digest being somewhat greater than that of the undigested RNA and restoring incorporation to the level attained in the presence of DNA (Gale, 1957). The activity of RNA digests is lost on dialysis, and fractionation by paper chromatography or ionophoresis leads to the separation of several active materials. Preliminary investigations showed that activity lies in a number of fractions, the most active of which possess properties indicating that they are not of a conventional nucleotide nature (Gale, 1957). Early attempts to separate the 'glycine-incorporation factor' consisted essentially of removing polynucleotides, nucleotides and nucleosides from the digested RNA, the active material remaining in the residues from the various treatments. In this way a preparation was made which had an activity some 10^4 times that of the RNA from which it was prepared when compared on a basis of optical density at 260 mμ; yields were too small to permit any characterization of the factor. In the course of the fractionations it appeared that there were a number of related 'incorporation factors' in the RNA digest, the factors differing in their R_F values in chromatography with propan-2-ol–aq. NH_3 soln. as solvent, in their acid lability and in the range of amino acids for whose incorporation they could replace nucleic acid. The aim of the present work has been to isolate one of these factors in larger quantities and to study some of its biochemical properties. Unfortunately it is present in trace amounts so that it has not been possible to isolate sufficient material to identify the nature of the factor. The method described below is adequate for the preparation of material for biological studies and is reported in order that other workers may be able to make and study the factor. The discussion of this paper is deferred until the end of the following paper by Gale & Folkes (1958).

METHODS

In general the procedures adopted have been those described by Gale & Folkes (1955*a*); such modifications as have arisen in the course of the work are as follows:

Preparation of disrupted cells. Staphylococcus aureus strain Duncan has been grown, harvested and washed as previously described (Gale & Folkes, 1955*a*). The washed cells (700–800 mg. dry wt.) are pelletted in a centrifuge tube and 0·5 ml. of 40% (w/v) sucrose in buffered saline is added (final sucrose concentration about 10%); the pellet is rubbed up in the buffered sucrose and transferred as quantitatively as possible to the cup of the transducer assembly. This provides optimum conditions for cell disintegration with the assembly at present in use (R. Davies, to be published). The assembly is cooled by a rapid stream of cold water, and supersonic vibration (25 kcyc./sec.) generated by a 500 w Mullard magnetostrictor oscillator applied for 20 min. The disintegrated material is then washed out of the cup with 10 ml. of 10% (w/v) sucrose in buffered saline and fractionated by centrifuging as previously described. The yield of disrupted cells is usually of the order of 20–50 mg. dry wt.

Buffered saline. The stock solution contained 0·1% KH_2PO_4, 0·33% Na_2HPO_4, 0·1% NaCl, 0·07% $MgSO_4$, $7H_2O$; pH was adjusted to 6·25. This solution was normally diluted, 1 part:2 parts of distilled water, for the disintegration medium, or 1:1 in the incubation medium.

Depletion of nucleic acid content of disrupted cells. The separated fraction of disrupted cells is suspended in 10 ml. of 0·1 M-phosphate (KH_2PO_4–Na_2HPO_4) buffer, pH 7·0, containing about 1 mg. of Streptodornase preparation (Lederle) and 3 mM-$MgSO_4$. The suspension is incubated for 1 hr. at 37°, centrifuged, the deposit resuspended in 10 ml. of buffered saline and 0·5 ml. (=1–2 mg. dry wt. of disrupted-cell material) added to each experimental tube.

Assay of activity of incorporation-factor preparations. Incubation mixtures are set up in 15 ml. centrifuge tubes as previously described (Gale & Folkes, 1955*a*, 1957); each tube contains 4 mg. of adenosine triphosphate, hexose diphosphate (≡30 mg. of neutral barium salt), 1·5 ml. of buffered saline, 0·2 μc of [^{14}C]glycine (generally labelled, specific activity 4·0 mc/m-mole), nucleic acid-depleted disrupted-cell preparation and additions as below; the final volume is adjusted to 3 ml. with water if necessary. The assay involves comparing the restoration of glycine incorporation brought about by the test material with that effected by an amount of staphylococcal nucleic acid which is sufficient to saturate the incorporation system. The latter quantity usually corresponds to 0·5–1·0 mg. of nucleic acid; minor variations occur between different nucleic acid preparations and the amount required to saturate glycine incorporation is determined experimentally for each new preparation; all preparations when present in sufficient amount give the same incorporation. Nucleic acid preparations are kept in solution in water at −15°. Each assay series involves (1) one tube containing no nucleic acid or other activator (this constitutes the control or basal incorporation measurement), (2) one tube containing an amount of nucleic acid known to saturate the incorporation system, and (3) a series of tubes containing graded amounts of the material to be assayed. Incorporation-factor preparations show optimum activity over a small range of concentration only and may be inhibitory at high concentrations (Gale, 1957); it is therefore necessary to test the preparations over a range of concentrations spaced at small intervals. In preparations made up as described below, the active range usually lies somewhere between 0·01 and 0·3 ml., and tests are first carried out with 0·01, 0·03, 0·1

and 0·3 ml. of solution. The tubes are incubated for 90 min. at 37°, precipitated with an equal volume of 10% (w/v) trichloroacetic acid, and washed and prepared for counting as previously described. The amount of test material which restores glycine incorporation to the level attained in tube (2) is determined from the results; this amount of factor has been termed 1 nucleic acid unit and the strength of solutions expressed as nucleic acid units/ml.

The activity of the disrupted-cell preparation varies from time to time, as does the degree of nucleic acid depletion obtained by the standard procedure (Gale & Folkes, 1955*a*, 1957), although results have been markedly more consistent since the adoption of the modifications recommended by R. Davies (in preparation). The incorporation of glycine by disrupted-cell preparations under the conditions described corresponds to 2000–3000 counts/min./mg. dry wt. (Gale & Folkes, 1957), and this can be reduced to a value between 50 and 500 counts/min./mg. by nucleic acid depletion. The success of the depletion can be gauged by the difference between the incorporation in tubes 1 and 2 above. In the best experiments, the value in tube 1 has been 3–5% of that in tube 2. Occasionally, for reasons as yet unknown, the difference is smaller. If incorporation in tube 1 is more than 40% of that in tube 2, then assays are unreliable and, in general, results are not accepted unless the value in tube 2 is at least three times that in tube 1. With the equipment used throughout this work, 1 μm-mole of [^{14}C]glycine gave 362 counts/min.

Chromatography. Two solvents have been used: 70% (v/v) propan-2-ol–30% (v/v) water, run in tanks containing ammonia vapour supplied from a shallow dish containing 1 ml. of aq. NH_3 soln. (sp.gr. 0·880)/l. tank volume; ether–ethanol–aq. NH_3 soln. (sp.gr. 0·880)–water (50:40:1:10, by vol.). Whatman paper grade no. 3 was used for most of the preparations, although the final stages of those preparations used for spectroscopic work were run on grade no. 2 papers which had been exhaustively washed (Connell, Dixon & Hanes, 1955) to remove background materials and were provided by Professor C. S. Hanes, F.R.S., and Dr F. Sanger, F.R.S.

Ionophoresis. Ionophoresis was carried out on strips (9 cm. × 50 cm.) of Whatman paper grade no. 3MM. The strip was soaked in 0·05M-carbonate (Na_2CO_3–$NaHCO_3$) buffer, pH 10·5, and excess of liquid blotted off with filter paper. The material for treatment (0·3–0·4 ml.) was streaked across the paper on a line 20 cm. from one end and the paper immediately immersed in carbon tetrachloride in the centre chamber of an ionophoresis apparatus; the ends of the paper were dipped into separate electrode vessels containing 0·05M-carbonate (Na_2CO_3–$NaHCO_3$) buffer, pH 10·5, and a potential of 1000v was applied across the electrodes. We are indebted to Dr R. Markham, F.R.S., for advice concerning the design and use of the equipment.

Bulk preparation of nucleic acid. Preparations of nucleic acid have been made from both *S. aureus* and *Bacillus megaterium*, and we are indebted to the Microbiological Research Establishment, Porton, Wilts. for providing us with approx. 500 g. of freeze-dried preparations of each of these organisms.

For disintegration of *S. aureus*, the freeze-dried material was rubbed up in 10% (w/v) buffered sucrose to form a 25% (w/v) suspension and this was treated in the supersonic assembly in 10 ml. batches. After disintegration for 15 min. each batch was diluted with 10 ml. of buffered sucrose and centrifuged to remove unbroken cells. The residues of unbroken cells were collected and exposed again to treatment. Supernatant extracts were accumulated and their nucleic acid was prepared in batches by the method of Jones (1953). Approx. 15 g. of nucleic acid was obtained from 400 g. dry wt. of *S. aureus* Duncan.

B. megaterium is broken by the supersonic treatment far more readily than *S. aureus* and it was possible to arrange disintegration on a continuous-flow basis. A 5% (w/v) suspension of dried *B. megaterium* in buffered saline was fed continuously through the supersonic assembly at a rate so controlled that the cells were exposed to vibration for 3–4 min. The effluent was collected and prepared in batches as was the staphylococcal preparation.

Enzyme synthesis. The development of catalase and β-galactosidase activities in the disrupted-cell preparation was studied as described by Gale & Folkes (1955*b*). Galactose (Kerfoot) was used as inducer of β-galactosidase synthesis.

EXPERIMENTAL AND RESULTS

Preparation of a glycine-incorporation factor

Preliminary work. The earlier studies which led to the discovery of incorporation factors (Gale, 1957) indicated that the 'glycine-incorporation factor' is reasonably stable to acid or alkali; soluble in ethanol; devoid of charge (as judged by electrophoretic mobility) in phosphate at pH 2·4, formate at pH 3·5, acetate at pH 5·5, or borate at pH 9·0; possesses a weak positive charge in carbonate buffer at pH 10·5; runs in propan-2-ol–aq. NH_3 soln. with R_F 0·6–0·7 and in propan-2-ol–HCl with R_F 0·9. The property which seemed most promising as a starting point for isolation was ethanol solubility. In the earlier work, the active material was subjected to electrophoresis in each of the buffers mentioned above, and the fraction positively charged at pH 10·5 eluted, dried and extracted with ethanol at room temperature. Extraction of dry nucleic acid or ribonuclease digest thereof by shaking at room temperature did not result in any activity passing into ethanol. However, it was found that extraction of freeze-dried digest in a Soxhlet apparatus resulted in activity passing into ethanol; purification of active material in terms of optical density at 260 mμ amounted to 300–1000 times.

Source of nucleic acid. Gale & Folkes (1955*a*) found that nucleic acid preparations from a number of sources other than *S. aureus* were unable to restore glutamic acid incorporation in nucleic acid-depleted disrupted staphylococcal cells. In the course of the present studies, a number of microbial cells have been tested as sources of nucleic acid preparations active in restoring glycine incorporation. Active preparations have been obtained from *Escherichia coli*, *B. megaterium* and from baker's yeast; in all cases the activity has been enhanced by digestion with ribonuclease. The procedure described below has been worked out with nucleic acids from *S. aureus* and *B. megaterium* as starting material but has also been applied successfully on a small scale to nucleic acid from baker's yeast, although it is not possible to say whether the factors isolated are the same for each source. *B. megaterium* appears to provide the best starting material, as this organism is disintegrated readily and the product appears to contain fewer impurities as judged by the appearance of the chromatograms made in

the course of the preparation. The method has also been applied to commercial-yeast nucleic acid, and some active material was obtained, but the yield was poor; the product was less pure than that from other sources and it lost activity rapidly. No differences have been observed in the biochemical activities of the incorporation-factor preparations from *S. aureus* and *B. megaterium*.

Stage 1*: digestion of nucleic acid.* Enzymic digestion of RNA does not affect the activity of the glycine-incorporation factor but renders it extractable. The procedure adopted has been that recommended by Dr R. Markham, F.R.S. (personal communication) for digestion to the mononucleotide stage with minimum destruction. The nucleic acid is dissolved to give about 5% (w/v) solution in 0·01 M-phosphate (KH_2PO_4–Na_2HPO_4), pH 7·0, and incubated overnight at 37° in the presence of 20 μg. of ribonuclease (Sigma Chemical Co.)/ml. After incubation, 2 N-NaOH is added to a final concentration of 0·1 N and incubation continued for a further 5 hr. The solution is then neutralized and frozen at −15° before evaporation to dryness.

Stage 2*: extraction with ethanol.* Activity can be extracted from nucleic acid digests in solution by shaking with 2-methylpropan-1-ol or in the dry state by continuous extraction with absolute ethanol or methanol. On a basis of optical density at 260 mμ, the purification attained by ethanol extraction is 10–30 times greater than that by butanol or methanol. Continuous extraction with ethanol in a Soxhlet apparatus has been adopted as a convenient procedure, the ethanol extract being collected and prepared every 48 hr. Extraction continues for 2–3 weeks. If extraction is continued to the stage when no further activity can be detected in the extract, then the ribonucleotide residues are also found to be inactive. In bulk preparations it has not been customary to remove DNA from nucleic acid; in such cases the residual nucleic acid and nucleotide mixture retains considerable activity after extraction has ceased to remove active material. If the DNA is now hydrolysed to the mononucleotide stage by incubation at pH 1 and 55° for 40 min. (D. B. Dunn, personal communication) further extraction is possible and can be continued until the nucleotide residues are completely inactive. Ethanol extracts are taken to dryness by distillation under reduced pressure and the residue is taken up in 0·05 M-carbonate buffer, pH 10·5; the residue from extraction for 48 hr. from 15 g. of nucleic acid is conveniently taken up in 5–6 ml. of buffer for the next stage. Accurate assay of factor in these extracts is frequently impossible as inhibitory material is present; this is particularly so in the material extracted during the first few days' treatment of a new digest. The inhibitory material is removed in stage 3.

Stage 3*: ionophoresis at* pH 10·5. The solution obtained at stage 2 is subjected to ionophoresis on paper as described above. The potential is applied for 3½–4 hr., and the paper then removed and dried in air. Active material is found in a band which has moved 5–10 cm. towards the cathode; the band can be located by its absorption of u.v. light of wavelength about 260 mμ, by exposing the paper in contact with photographic paper (Ilford reflex contact document no. 50 M), or by its light-blue fluorescence when examined in light emitted by a mercury-vapour lamp fitted with Woods glass envelope (Osram Mbw/v). The strip of paper containing the band is cut out, eluted with about 1 ml. of water, and the eluates are frozen at −15° and dried. Ultraviolet-light-contact photographs of papers show that large amounts of u.v.-absorbing materials pass towards the anode during the ionophoresis. Inhibitory substances are removed during this stage. The amount of u.v.-absorbing impurity decreases as the time of alcohol extraction increases. The early studies showed that some incorporation factors possess a negative charge at pH 10·5 and the yield of activity towards glycine is usually 30–50% of that in stage 2, although the inaccuracy of assay at stage 2 renders such calculations dubious.

After several ionophoretic runs, the electrode vessels frequently become contaminated with carbon from the electrodes. If such contamination occurs in the buffer of the cathode vessel, carbon particles move across the paper and tend to accumulate in the band containing active material; this can then be accurately located by the black or brown stain on the paper.

Stage 4*: second extraction with ethanol.* The eluates from stage 3 contain considerable quantities of sodium carbonate but the active factor can be readily extracted from the dry powder by ethanol. The combined eluates from eight to ten papers are dried and extracted once with 5 ml. of absolute ethanol at room temperature overnight, followed by two extractions of 15–20 min. by 5 ml. of hot ethanol. This treatment extracts 90–95% of the activity and the carbonate residues are discarded. The ethanol extracts are combined and evaporated to approx. 0·5 ml.

Stage 5*: chromatography in propan-2-ol–aq. ammonia soln.* The concentrated ethanol extracts from stage 4 are now streaked across the top of a large sheet of Whatman no. 3 paper and a descending chromatogram is developed in propan-2-ol–aq. NH_3 soln. until the solvent front has reached the bottom of the paper (50 cm. in 16–18 hr. at room temp.). The region R_F 0·85–1·0 is usually bright yellow while the paper remains wet with ammonia, and the limits of this yellow band are marked on the paper while still wet. The paper is now dried and examined under u.v. light; a narrow streak of light-blue fluorescence can usually be seen at R_F 0·60–0·65; this is marked on the paper. The bulk of the factor activity is found between this streak and the beginning of the yellow material, i.e. between R_F 0·65 and 0·85.

If a strip is cut from the side of the chromatogram and sprayed with 1% (w/v) iodine in ethanol (Cowgill, 1955), a strong purplish-brown band appears in the region R_F 0·5–0·6 and lighter stains in regions R_F 0–0·2 and 0·65–0·7. At one time it was thought that the last stain marked the active factor, but the degree of staining has been found to be very variable and it is no longer used as a reliable guide. Iodine staining and fluorescence in u.v. were used to detect the presence of materials on the paper and the chromatogram was divided into seven bands as shown in Table 1. These bands were cut out, each was eluted with about 5 ml. of water and the eluates were tested for activity of glycine-incorporation factor. Table 1 shows typical results obtained with preparations from *S. aureus* amd *B. megaterium*. The activity from the preparation from *B. megaterium* is found in a single band between R_F 0·75 and 0·85, whereas that from *S. aureus* is more widely distributed, the most active fraction occurring between R_F 0·6 and 0·75 with a significant activity also present in the iodine-staining material at R_F 0·5–0·6. The active material in the preparation from *B. megaterium* consistently runs somewhat faster than that from *S. aureus* and may not be identical with the latter.

Table 1. *Distribution of glycine-incorporation factor on chromatograms at stages 5 and 6*

Activity is expressed as nucleic acid units, as described in the text. (1) and (2) refer to different preparations.

Stage 5. Solvent—Propan-2-ol–aq. NH_3 soln.

		Nucleic acid units eluted			
		Staphylococcus aureus		*Bacillus megaterium*	
Band	R_F	(1)	(2)	(1)	(2)
A	0·9–1·0	0	0	0	0
B	0·85–0·9	10	0	0	0
C	0·75–0·85	25	15	198	420
D	0·6–0·75	100	45	24	15
E	0·5–0·6	30	13	0	0
F	0·3–0·5	0	6	0	0
G	0·0–0·3	10	5	10	28

Stage 6. Ethanol–ether–aq. NH_3 soln.

		Nucleic acid units eluted			
		Staphylococcus aureus		*Bacillus megaterium*	
Band	R_F	(1)	(2)	(1)	(2)
α	0·9–1·0	0	0	0	0
β	0·75–0·9	6	16	0	0
γ	0·6–0·75	80	127	70	210
δ	0·5–0·6	14	60	27	2
ε	0·2–0·5	4	0	0	0
ζ	0·0–0·2	0	0	0	0

With the staphylococcal preparation a small band of active material is sometimes obtained at R_F 0·2, but no studies have been made on this fraction. For large-scale preparations, it is convenient to cut the paper between the marks at R_F 0·65 and 0·85 as described above; the strip is eluted with 5–10 ml. of water and the eluate freeze-dried. The dry material appears as a yellow or red gum. The yield of activity over stage 5 may be 50% or less. Attempts have been made to account for this loss, but since no increase in activity has been obtained by combining eluates from all parts of the paper, or by increasing the time and volume of elution, it seems that inactivation must take place during the running of the chromatogram.

Stage 6: chromatography in ethanol–ether–aq. ammonia soln. The dried eluates from stage 5 are accumulated, dissolved in approx. 0·5 ml. of water and run on grade no. 3 paper in ethanol–ether–aq. NH_3 soln. solvent. Table 1 shows the separation obtained in typical runs of material from *S. aureus* or *B. megaterium*. The activity is found in both cases to run as a single band between R_F 0·6 and 0·75; the material from *B. megaterium* runs as a very narrow band corresponding to a width in R_F units of 0·05, whereas that from *S. aureus* tends to spread if the activity on the paper is greater than 200 nucleic acid units. No satisfactory way of marking the active material on the paper has been discovered and it must be located by assay of eluates from the region R_F 0·6–0·8. The yield of activity is quantitative. Eluates are freeze-dried and yield an amber gum. The activity of deep-frozen solutions decreases slowly on keeping; about 50% of the activity may be lost in 2–3 months' storage at −15°. The dry gum is more stable, but significant deterioration occurs over a period of 6 months; within the limited experience so far obtained the preparation from *B. megaterium* appears to be more stable than that from *S. aureus*.

Properties of the incorporation-factor preparation

The eluate obtained at stage 6 has been used for the studies described in the rest of this paper and the next one (Gale & Folkes, 1958). The yield in terms of nucleic acid units, assayed against glycine incorporation, is 15–20% of that in the nucleic acid starting material and the approximate purification on a dry-weight basis is about 1000 times. The u.v. spectrum of the stage 6 eluate is similar to that reported previously (Gale, 1957), but it now seems probable that this spectrum is due to impurities in the preparation. Preparations have been examined by Dr J. G. Buchanan, Chemistry Department, King's College, Newcastle-on-Tyne, who reports that colour reactions for aromatic amines or phenols are negative and that the factor is not held at all on Dowex 50 (Na^+). From the chemical point of view, the preparation from *B. megaterium* appears to be much cleaner than that from *S. aureus*, which contains many impurities. Chromatograms were run with 10% (w/v) Na_2CO_3 as solvent and assayed for activity; active material from the preparation from *B. megaterium* was found at R_F 0·75–0·8, whereas that from the preparation from *S. aureus* was found in the region of R_F 0·5. Material with a spectrum showing a peak at 343 mμ in alkali (see Gale, 1957) was found at R_F 0·5 on the chromatogram containing *B. megaterium* material, whereas eluates from the active section had no specific absorption between 240 and 360 mμ at pH 1 or 13 in the concentration available (20 nucleic acid units/ml.). Insufficient material has been obtained to carry out further purification or characterization at present.

Stabilities. Table 2 shows the loss of activity towards glycine incorporation when the preparation is exposed at 100° to acid or alkali. The incorporation factor is markedly more stable to alkali than to acid. Acid hydrolysis renders the preparation inhibitory. Gale & Folkes (1957) found that the inhibitory action of certain benzimidazole, quinoline and aminophenol derivatives can be antagonized by 6-amino-4-hydroxybenzotriazole; Table 3 shows that the same is true for the inhibitory action of the acid-hydrolysed incorporation-factor preparation.

Tests of known substances. The following substances have been tested for activity of glycine-incorporation factor over a wide range of concentrations (0·1 μM–0·1 mM): pyridoxin, pyridoxal,

Table 2. *Action of acid and alkali on the activity of the incorporation-factor preparation (stage 6)*

Glycine incorporation was studied under condition 1 (glycine being the only amino acid added to the incubation medium) in nucleic acid-depleted disrupted staphylococcal cells in the presence and absence of (*a*) 1 mg. of staphylococcal nucleic acid/3 ml. and (*b*) suboptimum concentration of incorporation-factor preparation subjected to treatment at 100° as below. Incorporation was estimated on trichloroacetic acid precipitate obtained after incubation for 90 min. at 37° and is expressed as counts/min./mg. of disrupted-cell preparation.

Additions to incubation mixture	Previous treatment at 100°		Glycine incorporation: Counts/min.	Glycine incorporation: Increase due to addition	Incorporation-factor activity as % of initial value
None	—		948	—	—
Nucleic acid	—		3699	2751	—
Incorporation-factor preparation	None		2490	1542	100
	N-HCl	30 min.	1462	514	33
		60 min.	675	(−273)	0
	0·1 N-HCl	30 min.	1997	1049	65
		60 min.	1451	503	33
		120 min.	1063	115	7
		240 min.	619	(−329)	0
	N-NaOH	60 min.	2497	1549	100
		120 min.	2209	1261	82
		180 min.	1688	740	48
		240 min.	920	(−28)	0

Table 3. *Antagonism of hydrolysed-factor preparation by 6-amino-4-hydroxybenzotriazole*

Glycine incorporation was studied under condition 1 in disrupted staphylococcal cells in the presence and absence of 1 mM-6-amino-4-hydroxybenzotriazole (AHBT) and a preparation of 3 nucleic acid units of incorporation factor/ml. was heated in N-HCl for 90 min. at 100°. Incorporation was estimated on trichloroacetic acid precipitate obtained after incubation for 90 min. at 37° and is expressed as counts/min./mg. of disrupted-cell preparation.

Vol. of hydrolysed factor preparation added/3 ml. (ml.)	Glycine incorporation: AHBT absent: Counts/min.	AHBT absent: % effect	AHBT present: Counts/min.	AHBT present: % effect
None	3273	—	3714	—
0·2	2220	−32	4033	+8
0·4	1623	−51	3535	−5

pyridoxamine, pyridoxal phosphate, pyridoxamine phosphate, biotin, vitamin B_{12}, 5:6-dimethylbenzimidazole, 5:6-dimethyl-1-β-D-ribofuranosylbenzimidazole, 5:6-dimethyl-1-α-D-ribofuranosylbenzimidazole, folic acid, *p*-aminobenzoic acid, rhizopterin, pteroic acid, pteroylaspartic acid, xanthopterin, leucopterin, 2-amino-4-hydroxypterin, 2-amino-4-hydroxy-6-carboxypterin, 2-amino-4-hydroxy-6-formylpterin, 2-amino-4-hydroxy-6-trihydroxypropylpterin, 2-amino-4-hydroxy-6-(2:3-dihydroxypropyl)pterin (biopterin), nicotinamide, dihydroxyphenylalanine, ribose, ribitol, arabitol, mannitol, kinetin (Miller, Skoog, Saltza & Strong, 1955), cycloadenosine (Clark, Todd & Zussman, 1951), 2-hydroxy-6-sulphydrylpurine, 2:5:6-triamino-4-styrylpyrimidine, adenosine, guanosine triphosphate, cytidine, mycobactin (Snow, 1954). Guanosine and uridine showed activity at 0·1 mM concentration (Gale, 1955), but these were preparations from natural sources and their activity is probably attributable to impurities. We are indebted to Drs E. Kodicek, A. Albert, E. L. Patterson, J. G. Buchanan, F. M. Strong, V. M. Clark, D. Raacke, F. Bergel and G. A. Snow for samples of some of the above substances.

Activation of the incorporation of amino acids by the factor preparation

Fig. 1 shows the ability of the preparation to replace nucleic acid for the incorporation of a variety of amino acids each studied under conditions 1 (i.e. the labelled amino acid being the only amino acid added to the incubation mixture). It can be seen that the preparation replaces nucleic acid completely for the incorporation of glycine, phenylalanine, aspartic acid, leucine, glutamic acid, arginine and lysine, but gives approximately 50% replacement for the incorporation of isoleucine, tyrosine, proline and valine. The preparation therefore activates a wider range of amino acids than the 'glycine-incorporation factor' previously described (Gale, 1957) and also differs from that

preparation in its greater sensitivity to acid. Fig. 1 shows, for glycine incorporation, that there is an optimum concentration of the factor, higher concentrations being markedly less effective. There would appear to be small differences in the concentration of factor optimum for the incorporation of different amino acids.

Fig. 2 shows the effect of the presence of factor on the incorporation of glutamic acid under conditions 1 and 2 (i.e. in the presence of the complete mixture of amino acids necessary for protein synthesis). It seemed possible that the inability of the factor preparation to replace nucleic acid for the incorporation of valine, isoleucine, proline and tyrosine might be a consequence of carrying out the experimental test under condition 1. Table 4

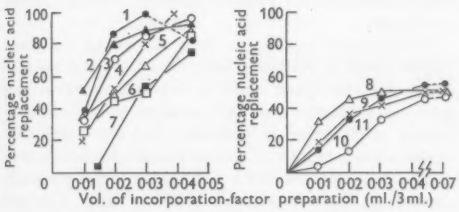

Fig. 1. Replacement of nucleic acid by the incorporation-factor preparation for the incorporation of amino acids into the trichloroacetic acid precipitate after 90 min. under condition 1. Disrupted staphylococcal cells are depleted of nucleic acid and incubated with each amino acid in turn under condition 1 in the presence of and absence of graded amounts of incorporation-factor preparation. Results obtained have been expressed as a percentage of the increase in incorporation obtained in the presence of 1 mg. of staphylococcal nucleic acid. Condition 1: incubation mixture contains ATP, hexose diphosphate, buffered saline and a single amino acid; amount of amino acid added corresponds to 0·2 μc of ^{14}C/3 ml. in each case. Amino acids: 1, glycine; 2, phenylalanine; 3, aspartic acid; 4, leucine; 5, arginine; 6, glutamic acid; 7, lysine; 8, isoleucine; 9, tyrosine; 10, proline; 11, valine.

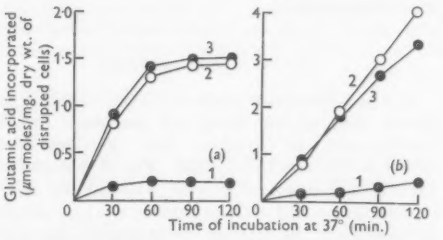

Fig. 2. Effect of the incorporation-factor preparation on the incorporation of glutamic acid into nucleic acid-depleted disrupted staphylococcal cells incubated under (*a*) condition 1 (glutamic acid being the only amino acid added to the medium) and (*b*) condition 2 (a complete mixture of the 18 amino acids necessary for protein synthesis was present). Condition 1: incubation mixture contains ATP, hexose diphosphate, buffered saline and 0·2 μc of [^{14}C]glutamic acid (11·3 mc/m-mole)/3 ml. Incorporation was determined on trichloroacetic acid precipitate; 1 μm-mole of glutamic acid≡1560 counts/min. Condition 2: incubation mixture as for condition 1 with addition of amino acid mixture (excluding glutamic acid) at final concentration of each component = 270 μg./ml. and glutamic acid diluted so that 1 μm-mole≡223 counts/min. Curve 1, control with no additions; curve 2, 1 nucleic acid unit of incorporation factor added/3 ml.; curve 3, 1 mg. of nucleic acid added/3 ml.

Table 4. *Replacement of nucleic acid by incorporation-factor preparation for the incorporation of isoleucine and valine*

Nucleic acid-depleted disrupted cells were incubated in the presence of adenosine triphosphate, hexose diphosphate and 0·20 μc of valine or isoleucine (generally labelled with ^{14}C) alone (condition 1) or in the presence of 0·1 ml. of amino acid mixture A/3 ml. [condition 2; amino acid mixture A = mixture of 18 natural amino acids necessary for protein synthesis (Gale & Folkes, 1955*a*)]. The labelled amino acid is omitted from the mixture A in each case. Additions are 1 mg. of staphylococcal nucleic acid or stated amounts of incorporation-factor preparation as used in Fig. 1. Counts were determined on trichloroacetic acid precipitate obtained after incubation for 90 min. at 37° and are expressed as counts/min./mg. dry wt. of disrupted cells.

Labelled amino acid	Additions to medium	Condition 1 Counts/min.	Condition 1 Increase on control	Condition 1 % effect of nucleic acid	Condition 2 Counts/min.	Condition 2 Increase on control	Condition 2 % effect of nucleic acid
Isoleucine	None (control)	412	—	—	320	—	—
	Nucleic acid	1903	1419	100	867	547	100
	Incorporation factor 0·03 ml.	683	271	18	570	250	45
	Incorporation factor 0·07 ml.	1108	696	47	624	304	55
	Incorporation factor 0·15 ml.	1006	595	40	486	166	30
Valine	None (control)	629	—	—	346	—	—
	Nucleic acid	3103	2474	100	1240	894	—
	Incorporation factor 0·03 ml.	1303	674	27	692	346	40
	Incorporation factor 0·07 ml.	1643	1014	41	873	495	55
	Incorporation factor 0·15 ml.	1821	1192	48	806	460	51

shows, however, that the degree of nucleic acid replacement by the factor is the same for either valine or isoleucine incorporation whether the tests are carried out under condition 1 or 2. It seems probable therefore that the nucleic acid preparation must provide a further component (such as the 'valine factor' previously described by Gale, 1957) necessary for optimum incorporation of these amino acids.

Specific enzyme synthesis

In a previous paper (Gale & Folkes, 1955*b*) it was shown that nucleic acids are necessary for the development of catalase and β-galactosidase activities by disrupted cells. Fig. 3 summarizes experiments designed to test whether the factor preparation could replace nucleic acid in enzyme development. In catalase development, the addition of factor has no significant stimulatory action in the presence or absence of nucleic acid. As previously demonstrated, nucleic acid has little or no effect on the formation of the inducible β-galactosidase but the addition of a mixture of purines and pyrimidines produces rapid enzyme development; again the presence of factor has no significant effect, although a small inhibition can be seen in the presence of purines and pyrimidines. In each case the factor has been tested over a range of 0·1–2·0 nucleic acid units, but no stimulation of enzyme development was observed. It is clear that the factor cannot replace nucleic acid in these syntheses.

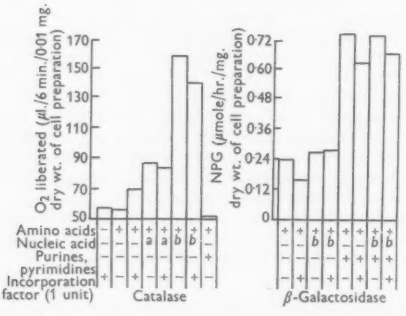

Fig. 3. Development of catalase and β-galactosidase activities in nucleic acid-depleted disrupted staphylococcal cells. Disrupted staphylococcal cells are depleted of nucleic acid and incubated at a final concentration of approx. 0·3 mg. dry wt./ml. in a medium containing ATP, hexose diphosphate, buffered saline, amino acid mixture A at a final concentration of 270 μg. of each component/ml. and other substances as shown. Staphylococcal nucleic acid was added at a concentration of (*a*) 0·1 mg. and (*b*) 0·3 mg./3 ml.; incorporation factor was added at 1 nucleic acid unit/3 ml.; purine–pyrimidine mixture was added to a final concentration of 100 μg. each of adenine, guanine, uracil and thymine/3 ml. Galactose as inducer of β-galactosidase was added to final concentration 2% (w/v). Conditions and tests were as in Gale & Folkes (1955*b*). Incubation time: 90 min. for catalase development, 120 min. for β-galactosidase development. Catalase activity is expressed as μl. of O_2 liberated in 6 min. by 0·01 mg. dry wt. of disrupted-cell preparation (initial wt.); β-galactosidase activity is expressed as μmoles of *o*-nitrophenyl β-galactoside (NPG) hydrolysed/hr./mg. dry wt.

Nucleic acid synthesis. It is shown in the next paper (Gale & Folkes, 1958) that the synthesis of RNA by disrupted cells is dependent upon the presence of factor and the question arises why the factor preparation is not essential for β-galactosidase development in the presence of purines and pyrimidines where RNA formation is presumably occurring (Gale & Folkes, 1955*b*). Table 5 shows that adenine incorporation into nucleic acid (Gale & Folkes, 1958) is dependent upon the presence of factor in the absence of galactose but that, in the presence of galactose, the incorporation is much greater and independent of added factor. The amount of galactose necessary to induce β-galactosidase is high and may well introduce active impurities; Table 5 also shows that the same amount of galactose introduced into the incubation medium for glycine incorporation under condition 1 produces a marked stimulation of that incorporation. It seems probable that the absence of any stimulation of β-galactosidase formation by added factor is due to the presence of activating substances in the galactose added as inducer, and also to synthesis of factor by the cells under the complex incubation conditions necessary for formation of β-galactosidase.

SUMMARY

1. Removal of nucleic acid from disrupted staphylococcal cells results in a decrease of up to 98% of the incorporation of glycine by the preparation. The incorporation can be restored by the addition of staphylococcal ribonucleic acid, or a ribonuclease digest thereof, to the incubation mixture. The restoration is brought about by the presence of trace components, called 'incorporation factors', in the digest.

2. A method for the separation and concentration of an incorporation factor is described.

3. The incorporation-factor preparation can replace nucleic acid for the incorporation of glycine, phenylalanine, aspartic acid, leucine, glutamic acid, arginine and lysine but gives only partial replacement for the incorporation of valine, isoleucine, tyrosine and proline.

Table 5. *Effect of galactose on adenine and glycine incorporation*

Adenine incorporation: nucleic acid-depleted disrupted cells were incubated in the presence of adenosine triphosphate (ATP), hexose diphosphate, a complete mixture of amino acids A, a mixture containing guanine, uracil, thymine and ^{14}C-labelled adenine as for optimum adenine incorporation into nucleic acid (Gale & Folkes, 1958). Galactose was added where shown below to a final concentration of 2% (w/v) as for β-galactosidase formation. Adenine incorporation was determined on the trichloroacetic acid precipitate obtained after incubation for 90 min. at 37° and is expressed as counts/min./mg. dry wt. of disrupted cells.

Glycine incorporation: nucleic acid-depleted disrupted cells were incubated under condition 1 in the presence of [^{14}C]-glycine, ATP and hexose diphosphate with and without nucleic acid (1 mg.) or 1 nucleic acid unit of incorporation factor. Glycine incorporation was estimated on the trichloroacetic acid precipitate obtained after incubation for 90 min. at 37° and is expressed as counts/min./mg. dry wt. of disrupted cells. Galactose was added where shown to a final concentration of 2% (w/v). +, Added; −, not added.

	Components of incubation mixture					
Labelled component	Galactose	Amino acid mixture	Purine–pyrimidine mixture	Nucleic acid	Incorporation factor	Incorporation (counts/min.)
Adenine	−	+	+	−	−	479
Adenine	−	+	+	−	+	3 291
Adenine	+	+	−	−	−	5 773
Adenine	+	+	−	−	+	5 892
Adenine	+	+	+	−	−	10 809
Adenine	+	+	+	−	+	10 848
Glycine	−	−	−	−	−	224
Glycine	−	−	−	+	−	2 134
Glycine	−	−	−	−	+	2 241
Glycine	+	−	−	−	−	1 487
Glycine	+	−	−	+	−	2 821

4. The incorporation-factor preparation does not replace, or enhance the activity of, nucleic acid for promotion of the development of catalase activity in nucleic acid-depleted disrupted cells. Nor does the factor preparation replace the mixture of purines and pyrmidines necessary for the formation of β-galactosidase in such preparations.

We would like to express our gratitude to Dr R. Davies, whose skilled and patient nursing of the supersonic generator has made this work possible.

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The Assimilation of Amino Acids by Bacteria

26. THE INCORPORATION OF ADENINE INTO THE NUCLEIC ACID OF DISRUPTED STAPHYLOCOCCAL CELLS

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Gale & Folkes (1953*a*, *b*) showed that there is an increase in the nucleic acid content of washed suspensions of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) when these are incubated in the presence of glucose, purines, pyrimidines and a mixture of all those amino acids necessary for protein synthesis. Omission of an essential amino acid resulted in cessation of nucleic acid synthesis. In the presence of the amino acid mixture, protein synthesis also occurred; the addition of a growth-limiting concentration of chloramphenicol gave rise to complete inhibition of protein synthesis but stimulated formation of ribonucleic acid (RNA). A number of investigations with amino acid-requiring mutants has since confirmed that nucleic acid synthesis in bacteria will take place only if all the amino acids required for protein synthesis are either present in the medium or can be synthesized by the organism (Borek, Ryan & Rickenbach, 1955; Gros & Gros, 1956; Pardee & Prestidge, 1956). Pardee, Paigen & Prestidge (1957) and Neidhardt & Gros (1957) have confirmed that RNA synthesis will occur in the presence of chloramphenicol but have shown that the RNA formed under these conditions differs from 'normal' RNA in electrophoretic mobility, molecular weight and stability.

Previous papers of this series (Gale & Folkes, 1955, 1958) have shown that removal of nucleic acid from disrupted staphylococcal cells markedly reduces the ability of the preparation to incorporate amino acids and that this ability can be restored by the addition to the incubation medium of deoxyribonucleic acid (DNA), RNA or an 'incorporation factor' prepared from digests of nucleic acid. The present communication describes the conditions necessary for the incorporation of labelled purines into the nucleic acid fraction of disrupted cells, and the role of nucleic acids and the incorporation factor in such processes.

METHODS

The methods for the growth, harvesting and disruption of *Staphylococcus aureus* strain Duncan, and the depletion of nucleic acid in the disrupted-cell fraction, have been described by Gale & Folkes (1955, 1958).

Incubation mixtures. Except where otherwise stated, incubations were carried out at 37° in stationary 15 ml. centrifuge tubes as described by Gale & Folkes (1955). In addition to 1·5 ml. of buffered saline, 4 mg. of adenosine triphosphate (ATP) and hexose diphosphate ≡ 30 mg. of neutral barium salt, the incubation medium contained 0·1 ml. of the amino acid mixture A, 0·1 ml. of a purine-pyrimidine mixture (PP) containing 1 mg. each of guanine, uracil and thymine/ml., 0·1 ml. of ribose (1 mg./ml.) and water to a final volume of 3 ml. An appropriate amount of either [8-^{14}C]adenine (specific activity 9·7 mc/m-mole) or [8-^{14}C]guanine (specific activity 3·3 mc/m-mole) was added; with the latter, guanine was omitted from the PP mixture. The counting equipment used throughout this work was calibrated against the adenine preparation: 1 μm-mole gave 1320 counts/min.

Nucleic acids. Nucleic acid was prepared from disintegrated *S. aureus* by the method of Jones (1953) and fractionated into DNA and RNA as previously described (Dutta, Jones & Stacey, 1953; Gale & Folkes, 1955).

Incorporation factor. The preparation used in the work described below was obtained from *Bacillus megaterium* nucleic acid as described by Gale & Folkes (1958).

Digests of nucleic acids. RNA was incubated at a final concentration of 5 mg./ml. in 0·05 M-phosphate (Na_2HPO_4–KH_2PO_4) buffer, pH 7·0, with 10 μg. of ribonuclease (Sigma Chemical Co.)/ml. overnight at 37°. The digest was used without further treatment. DNA was incubated under similar conditions but with 10 μg. of Streptodornase (Lederle)/ml. in the presence of 3 mM-$MgSO_4$; the deoxyribonuclease became inactive if the digest was kept at 4° for 3 days before use.

Preparation of fractions for counting. After incubation 3 ml. of cold 10% (w/v) trichloroacetic acid was added to each tube and the contents were cooled and held at 4° for 30 min. The precipitate was then centrifuged down, washed once with cold 5% (w/v) trichloroacetic acid, once with cold 5% (v/v) acetic acid and finally once with cold 1% (v/v) acetic acid. This precipitate is referred to as the 'trichloroacetic acid precipitate' and was prepared for counting as previously described (Gale & Folkes, 1953*c*). For estimating the proportion of activity in the RNA and DNA fractions, the precipitate was treated by a modification of the method of Schmidt & Thannhauser (1945): each sample was prepared in triplicate; one part (*a*) was counted as the trichloroacetic acid precipitate; a second part (*b*) was extracted at 90° for 15 min. with 5% trichloroacetic acid and the residue counted; the third part (*c*) was dissolved in 2 ml. of N-NaOH and incubated overnight at 37°, then neutralized, made N with respect to $HClO_4$, and cooled and held at 4° for 30 min.; the precipitate was centrifuged

down, washed once with cold 1% (v/v) acetic acid and prepared for counting. The activity of (*c*) gives a measure of incorporation into the DNA fraction, and $(a)-(b+c)$ gives a measure of incorporation into the RNA fraction.

Radioautography. To determine the distribution of radioactivity amongst the components of nucleic acid, the following procedure was adopted. The nucleic acid–protein fraction was precipitated after incubation by the addition of an equal volume of cold 10% trichloroacetic acid and the precipitate washed as above; the washed precipitate was extracted for 15 min. at 90° with 5% trichloroacetic acid, centrifuged down and the supernatant collected. The supernatant was extracted three times with an equal volume of ether to remove trichloroacetic acid and then freeze-dried. The dry material was taken up in 1 ml. of N-HCl and hydrolysed at 105° for 1 hr. It was again freeze-dried, the material taken up in 10–20 μl. of water and spotted on to Whatman no. 1 paper. The chromatogram was developed in propan-2-ol–HCl solvent (Markham & Smith, 1952) and the bases were located and identified by their ultraviolet absorption. The dried chromatogram was placed in contact with X-ray-sensitive film and radioactivity located by darkening of the film in the usual way.

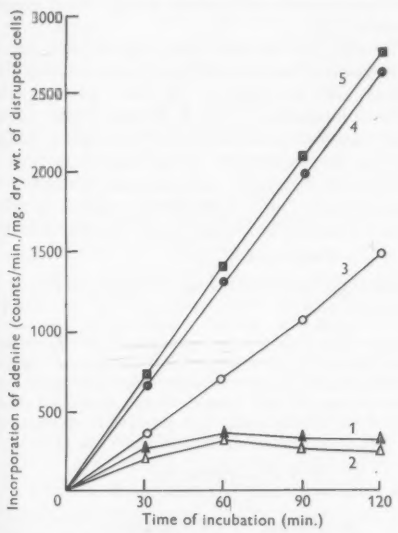

Fig. 1. Time course of the incorporation of adenine by disrupted staphylococcal cells. Disrupted-cell preparation was incubated at 37° in buffered saline at pH 6·2 containing, in 3 ml., 0·1 ml. of 4% adenosine triphosphate, 0·3 ml. of hexose diphosphate (≡30 mg. of neutral barium salt) and 0·017 mM-[8-^{14}C]adenine (specific activity 9·7 mC/m-mole) with additions as follows: curve 1, no addition; curve 2, 100 μg. of glutamic acid/ml. or 0·1 ml. of mixture PP containing 1 mg. each of guanine, uracil and thymine/ml.; curve 3, 0·1 ml. of amino acid mixture A containing 4 mg. each of 18 natural amino acids/ml.; curve 4, 0·1 ml. of amino acid mixture A + 0·1 ml. of mixture PP; curve 5, as curve 4 + 0·1 ml. of ribose (1 mg./ml.). Incorporation was determined on trichloroacetic acid precipitates and expressed as counts/min./mg. dry wt. of disrupted-cell preparation.

RESULTS

Conditions for the incorporation of [^{14}C]*adenine.* Fig. 1 shows the time course for incorporation of radioactivity into the trichloroacetic acid precipitate of disrupted cells incubated with [^{14}C]adenine. When incubated in the absence of the amino acid mixture A, a small incorporation takes place during incubation for the first 30 min. only. This incorporation is not significantly affected by the presence of PP, ribose or single amino acids such as glutamic acid, glycine or leucine. In the presence of the amino acid mixture A, incorporation follows a linear course for the 2 hr. experimental period; the rate of this incorporation is markedly increased by the addition of PP but little affected by the presence or absence of ribose. Table 1 shows the effect on the rate of incorporation of varying the concentration of amino acid mixture A or of adenine or guanine. Very similar results are obtained for incorporation into cells incubated with [^{14}C]guanine, although then the addition of [^{12}C]-adenine results in a marked decrease in incorporated radioactivity. Fig. 2 shows the effect of adenine concentration on the rate of incorporation under otherwise optimum conditions; the Michaelis constant is 4·3 μm-moles/ml. Unless otherwise stated, [^{14}C]adenine was used in the experiments described below at a final concentration of 17 μm-moles/ml. and guanine at 50 μm-moles/ml.

Nature of the radioactive material incorporated. When the disrupted-cell preparation is incubated with the complete mixture containing the amino

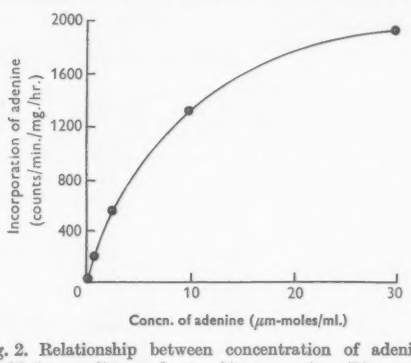

Fig. 2. Relationship between concentration of adenine added to medium and rate of incorporation. Disrupted-cell preparation was incubated as for curve 5, Fig. 1, and incorporation was estimated on trichloroacetic acid precipitates obtained after incubation at 37° for 1 hr.

acid mixture A, PP, ribose and [^{14}C]adenine, the incorporation of adenine is paralleled by an increase in nucleic acid as measured by the optical density at 260 mμ of the hot perchloric acid extract from the cold trichloroacetic acid precipitate of the preparation. However, since this increase represents only some 10–20 % increase on the initial value, it is not possible to obtain accurate measurement of nucleic acid synthesis by this means. Table 1 shows that the radioactivity of the cold trichloroacetic acid precipitate is quantitatively extracted by 5 % trichloroacetic acid at 90° in 15 min. whether incubation has taken place with ^{14}C-labelled adenine or guanine. Hydrolysis of the nucleic acid fraction and radioautography of the products has shown that the radioactivity of preparations incubated with [^{14}C]adenine, amino acid mixture A, PP and ribose resides solely in adenine; that of preparations incubated with [^{14}C]guanine, amino acid mixture A, pyrimidines and ribose resides almost completely in guanine with less than 1 % of the total activity in the adenine spot. Under these conditions therefore the radioactivity of the trichloroacetic acid precipitate can be taken as a measure of the incorporation into nucleic acid of the labelled purine added to the incubation mixture. Incorporation is expressed below in terms of counts/min., as no accurate measure of the dilution of added [^{14}C]adenine by metabolic adenine from added ATP has been obtained nor knowledge of the constancy of such dilution under the various experimental conditions used. Nucleic acid has been prepared from disrupted cells after incubation in the presence of [^{14}C]adenine under optimum conditions for incorporation, as above, and fractionated into RNA and DNA; in 10 samples the proportion of the radioactivity in the DNA fraction was 9·0–13·2, mean 12·2 %.

Effect of nucleic acid depletion. Removal of nucleic acid from the disrupted-cell preparation (Gale & Folkes, 1955, 1958) results in a marked decrease in the rate of adenine or guanine incorporation; the rate may fall to 3 % of that in the unextracted preparation. Fig. 3 shows the effect on the rate of incorporation of [^{14}C]adenine of adding staphylococcal nucleic acid to the incubation mixture. Fig. 4 shows that both RNA and DNA are effective in promoting the incorporation; their effects are nearly additive at suboptimum concentrations only.

The effect of nucleic acid in promoting the incorporation of amino acids can be reproduced if the nucleic acid is replaced by the 'incorporation-factor' preparation (Gale & Folkes, 1958). In the same way incorporation factor can replace nucleic acid for the restoration of adenine or guanine incorporation in nucleic acid-depleted disrupted-cell preparations. Table 2 shows the extent to which nucleic acid can be replaced by 1 nucleic acid unit

Table 1. *Conditions affecting the rate of incorporation of purines into the nucleic acid fraction of disrupted staphylococcal cells*

Disrupted-cell preparation was incubated at 37° in buffered saline at pH 6·2 containing (3 ml.) 0·1 ml. of 4 % adenosine triphosphate, 0·3 ml. of hexose diphosphate (≡30 mg. of neutral barium salt) and either (*a*) 0·017 mM-[8-^{14}C]adenine (specific activity 9·7 mc/m-mole) or (*b*) 0·05 mM-[8-^{14}C]guanine (specific activity 3·3 mc/m-mole) with additions as shown below. Amino acid mixture A = mixture of 18 natural amino acids at 4 mg. of each component/ml.; unlabelled guanine and adenine solutions contained 1 mg./ml. Counts were determined on trichloroacetic acid precipitate obtained after incubation for 90 min. Nucleic acid extraction was carried out in 5 % (w/v) trichloroacetic acid at 90° for 15 min.

Additions to incubation mixture			Rate of incorporation (counts/min./mg. of cells/90 min.)	
Amino acid mixture A (ml./3 ml.)	Guanine (ml./3 ml.)	Adenine (ml./3 ml.)	[^{14}C]Adenine supplied	[^{14}C]Guanine supplied
0	0·1	0	345	—
0·01	0·1	0	1530	—
0·03	0·1	0	1676	—
0·1	0·1	0	2021	—
0·3	0·1	0	1758	—
0	0	0	—	321
0·01	0	0	—	2031
0·1	0	0	1044	2950
0·3	0	0	—	2848
0·1	0·01	0	1360	—
0·1	0·03	0	1818	—
0·1	0	0·03	—	466
0·1	0	0·1	—	353
Residue remaining after nucleic acid extraction				
0·1	0·1	0	2	—
0·1	0	0	—	4

(Gale & Folkes, 1958) of factor for the incorporation of adenine. In the presence of the complete amino acid mixture A, whether PP and ribose are present or not, incorporation factor will completely replace nucleic acid. However, incorporation factor is less than half as effective as nucleic acid if amino acids are omitted or present in 'incomplete' mixtures in the medium; increasing the concentration of incorporation factor does not increase the degree of replacement.

Table 2 shows also that the addition of chloramphenicol to a medium containing amino acid mixture A, PP and ribose again renders incorporation factor incapable of replacing nucleic acid. In the experiment quoted in Table 2, the basal rate of incorporation is less than 3% of that obtained in the presence of added nucleic acid and it is under such conditions that chloramphenicol decreases the

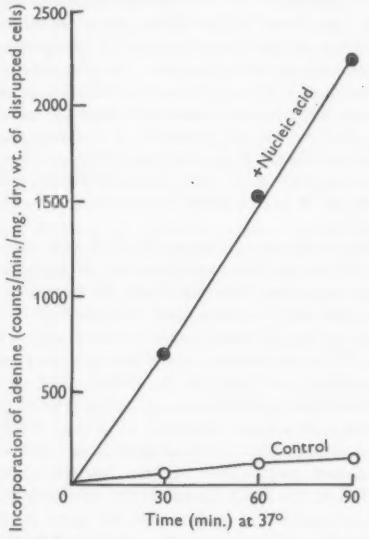

Fig. 3. Effect of nucleic acid on the incorporation of adenine by nucleic acid-depleted disrupted staphylococcal-cell preparation. Nucleic acid-depleted cell preparation was incubated as for curve 5, Fig. 1, in the presence and absence of 1 mg. of staphylococcal nucleic acid/3 ml. Incorporation was estimated on trichloroacetic acid precipitates.

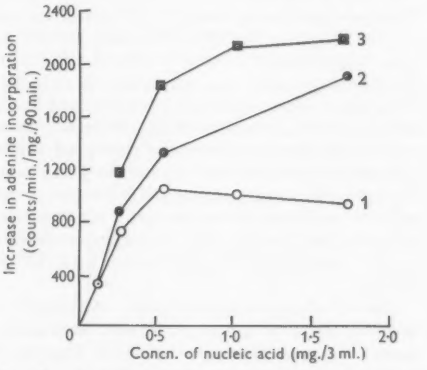

Fig. 4. Stimulation of adenine incorporation in nucleic acid-depleted disrupted cells by RNA and DNA. Conditions were as for Fig. 3; incorporation was estimated on trichloroacetic acid precipitate obtained after incubation for 90 min. Incorporation in control = 473 counts/min./mg. dry wt. of disrupted-cell preparation. Curve 1, RNA added; curve 2, DNA added; curve 3, RNA + DNA added.

Table 2. *Replacement of nucleic acid by the incorporation-factor preparation for adenine incorporation by nucleic acid-depleted disrupted cells*

Nucleic acid-depleted disrupted cells are incubated for 90 min. at 37° in buffered saline with ATP, hexose diphosphate (HDP) and [8-^{14}C]adenine as in Table 1. Additions were as shown below. Amino acid mixture = amino acids of mixture A less glycine, alanine and lysine; each component was present at final concentration 133 μg./ml. Mixture PP = guanine, uracil, thymine and ribose, final concentration 33 μg. of each component/ml. Final concentration of nucleic acid = 0·3 mg./ml.; chloramphenicol (CAP) = 30 μg./ml.; incorporation factor = 1 nucleic acid unit/3 ml. Adenine incorporation was estimated on the trichloroacetic acid precipitate and expressed as counts/min./mg. dry wt. of disrupted-cell preparation. +, Added; −, not added.

Components of incubation medium					[^{14}C]Adenine incorporated			
ATP HDP	Amino acid mixture	Glycine Alanine Lysine	Mixture PP	CAP	Basal	Nucleic acid added	Incorporation-factor preparation added	Replacement of nucleic acid (%)
+	−	−	−	−	29	614	340	45
+	−	+	−	−	44	643	262	27
+	+	−	−	−	57	1086	544	47
+	+	+	−	−	50	1576	1631	104
+	+	+	+	−	58	2923	2865	98
+	+	+	+	+	83	3564	1582	44

effectiveness of incorporation factor; if the basal rate is 10 % or more of the rate in the presence of nucleic acid, then chloramphenicol has little or no effect on the replacement of nucleic acid by incorporation factor. It would appear that, under conditions of effective depletion, the addition of nucleic acid to the incubation medium supplies some component necessary for the incorporation of adenine in the presence of chloramphenicol, and that this component is not supplied by either the incubation medium or the incorporation-factor preparation.

Nucleic acid preparations contain RNA and DNA and it has been shown above that some 12 % of the adenine incorporated under optimum conditions is found in the DNA fraction; repetition of these experiments in the presence of chloramphenicol shows that the proportion is reduced by 50 % (nine tests; mean reduction of count in DNA fraction = 50 %, range 35–70 %). When nucleic acid is partially removed from the disrupted cell and replaced by incorporation factor, the proportion of incorporated adenine in the DNA fraction is again reduced, as shown below, so that a combination of incorporation factor and chloramphenicol can result in insignificant incorporation into the DNA fraction.

Role of deoxyribonucleic acid. Although considerable error is involved in the estimation of counts incorporated into the DNA fraction, the results discussed above suggest that the disrupted cell is unable to synthesize DNA adequately in the presence of chloramphenicol. Fig. 4 shows that adenine incorporation in nucleic acid-depleted cells can be stimulated by the addition of DNA; this stimulation therefore can be used as a test for DNA synthesis. Table 3 shows the stimulation of adenine incorporation in a partially depleted disrupted-cell preparation by addition of DNA, RNA and digests thereof prepared by incubation with the appropriate nucleases. It can be seen that digestion of RNA has little or no effect upon its ability to stimulate adenine incorporation in the presence or absence of chloramphenicol. On the other hand, digestion of DNA decreases its ability to stimulate adenine incorporation and the digest is markedly less effective in the presence of chloramphenicol. These results are in accord with the suggestion that DNA plays a role in adenine incorporation and that synthesis of active DNA is inhibited by chloramphenicol.

Table 4 shows the effect of DNA and incorporation factor on the incorporation of adenine in a highly depleted disrupted-cell preparation incubated in the presence and absence of chloramphenicol. In this case, 1 unit of incorporation factor gave 70 % replacement of the stimulation produced by nucleic acid, and in the presence of chloramphenicol this replacement was reduced to 39 %. The presence of a small amount of added DNA gave little stimulation of incorporation, although a significant proportion of this incorporation was located in the DNA fraction; the addition of DNA and incorporation factor together gave a marked stimulation of incorporation, greater than the sum of the separate stimulations, and a significant in-

Table 3. *Stimulation of adenine incorporation by ribonucleic and deoxyribonucleic acids and their digests in the presence and absence of chloramphenicol*

Disrupted cells were partially depleted of nucleic acid and incubated for 90 min. at 37° in buffered saline containing ATP, hexose diphosphate, amino acid mixture A, purine and pyrimidine mixture PP and [8-^{14}C]adenine as in Table 1. Nucleic acids were added at a concentration of 0·5 mg./3 ml. Digests were prepared by overnight incubation at 37° and at pH 7·0 with corresponding nuclease, and added at the same concentration as nucleic acid. Incorporation was determined on trichloroacetic acid precipitates and expressed as counts/min./mg. dry wt. of disrupted-cell preparation. Basal rate of incorporation (in the absence of added nucleic acid) = 641 (A, chloramphenicol absent) or 1226 (B, 30 μg. chloramphenicol present/ml.). –, Not examined.

	Increase in incorporation of adenine due to addition of							
	A				B			
	DNA	RNA	DNA + RNA	DNA + RNA digest	DNA	RNA	DNA + RNA	DNA + RNA digest
(*a*) Nucleic acid stimulation	655	668	970	1327	1031	446	1276	1104
(*b*) Repeat of (*a*) after digestion of DNA	415	–	864	1014	248	–	380	675
% loss of stimulation after digestion of DNA	36	–	10	24	76	–	70	39
(*c*) Repeat of (*a*) after digestion of RNA	–	556	1327	–	–	735	1104	–
% loss of stimulation after digestion of RNA	–	17	0	–	–	0	13	–

corporation occurred in the DNA fraction. In the absence of chloramphenicol the effect of DNA and incorporation factor together was greater than that obtained with total nucleic acid; in the presence of the antibiotic, DNA + incorporation factor gave the same stimulation as total nucleic acid but there was no significant incorporation into the DNA fraction under these conditions.

Attempts have been made to obtain more marked effects of the action of DNA on the response to incorporation factor by exposing cells to longer periods (30 min.) of supersonic vibration and longer incubation (2 hr.) with deoxyribonuclease, but the resulting preparations have had very low activities which were not significantly stimulated by incorporation factor with or without DNA.

Inhibitors of incorporation of purine. Table 5 shows the action of certain inhibitors on the incorporation of adenine or guanine. The substances tested include many found to be inhibitors of incorporation of glycine (Gale & Folkes, 1957) and results with glycine incorporation under condition 2 are included for comparison. In general the substances tested seem to have significantly the same action on the incorporation of adenine, guanine or glycine.

Table 4. *Effect of incorporation factor and deoxyribonucleic acid on adenine incorporation in nucleic acid-depleted disrupted-cell preparation in the presence and the absence of chloramphenicol*

Highly depleted disrupted-cell preparation was incubated for 90 min. at 37° in buffered saline containing ATP, hexose diphosphate, amino acid mixture A, purine and pyrimidine mixture PP and [8-^{14}C]adenine as in Table 1. Total nucleic acid (1 mg.), DNA (0·1 mg.), incorporation factor (1 unit) and chloramphenicol (100 μg.) were added/3 ml. as shown below. Adenine incorporation was estimated on the trichloroacetic acid precipitate and fractionated into DNA and RNA as described in the text, and expressed as counts/min./mg. dry wt. of cell preparation. +, Added; −, not added.

Additions to incubation medium				Adenine incorporation (counts/min./mg.)	
Total nucleic acid	Incorporation factor	DNA	Chloramphenicol	DNA fraction	RNA fraction
−	−	−	−	8	46
+	−	−	−	86	1072
−	+	−	−	15	736
−	−	+	−	33	184
−	+	+	−	180	2301
−	−	−	+	18	94
+	−	−	+	20	1661
−	+	−	+	14	667
−	−	+	+	17	464
−	+	+	+	20	1597

Table 5. *Inhibitors of the incorporation of adenine, guanine and glycine into the trichloroacetic acid precipitate of disrupted staphylococci*

Disrupted staphylococcal cells were incubated for 90 min. at 37° in the presence of ATP, hexose diphosphate, amino acid mixture A, 0·017 mM-adenine, 0·05 mM-guanine, as in Table 1. In separate experiments, adenine, guanine or glycine is labelled with ^{14}C to specific activities 9·7, 3·3 or 2·0 mc/m-moles respectively. Tests are carried out in the presence and absence of inhibitors as indicated below and % inhibition is calculated from the counts/min./mg. of the trichloroacetic acid precipitate.

Inhibitor	Log molar concn.	% inhibition of incorporation: Adenine	Guanine	Glycine
1:2-Dichloro-5-nitro-4-(*p*-nitrobenzenesulphonamide)benzene	−6·0	60	66	68
Ethidium bromide	−5·0	65	—	51
8-Hydroxyquinoline	−4·0	84	79	56
4-Methoxy-6-nitrobenzotriazole	−4·0	69	76	36
4:5:6-Trichlorobenzimidazole	−4·0	17	30	40
4:5:6-Trichloro-1-α-D-ribofuranosylbenzimidazole	−4·0	32	7	40
4:5:6-Trichloro-1-β-D-ribofuranosylbenzimidazole	−4·0	28	7	25
1-Allyl-5:6-dimethylbenzimidazole	−4·0	6	15	8
5:6-Dimethylbenzimidazole	−3·0	26	45	52
5:6-Dimethyl-1-β-D-ribofuranosylbenzimidazole	−3·0	12	0	0
Puromycin nucleoside	−3·0	9	7	—
Aminopterin	−3·5	55	—	35

DISCUSSION

The incorporation of adenine into the nucleic acid fraction of disrupted staphylococcal cells is reduced almost completely by removal of nucleic acid from the cell preparation. If the nucleic acid depletion is partial, adenine incorporation can be fully restored by addition of the 'incorporation-factor' preparation to the incubation mixture, but if the depletion is great, then the factor preparation is less effective but its action can be markedly stimulated by the further addition of DNA. If depletion is great so that DNA is reduced to a low level in the preparation, no DNA is added and its synthesis prevented by the presence of chloramphenicol, then incorporation factor is less efficient than total nucleic acid in promoting adenine incorporation, and incorporation takes place almost exclusively into the RNA fraction. These results are consistent with the suggestion that DNA plays a role in the synthesis of RNA. It also appears from Table 4 that the presence of DNA may be necessary for DNA synthesis but the experimental values are too small for this deduction to be made with any certainty.

The results described in this paper and the preceding one (Gale & Folkes, 1958) show that nucleic acid preparations contain a trace component possessing highly significant activities in amino acid-incorporation reactions and RNA synthesis. It is not yet possible to say whether this 'incorporation factor' forms a component of nucleic acid or whether it occurs as an impurity adsorbed on to the polynucleotide. Unfortunately it has not yet been possible to characterize the factor, although tests have established that it is not a conventional nucleotide or derivative thereof. In the absence of knowledge concerning its chemical nature, its function can only be discussed in experimental terms. Removal of nucleic acid from the disrupted staphylococcal cell preparation is followed by decrease in the following processes: (1) incorporation of amino acids under condition 1 or 2 (Gale & Folkes, 1955, 1958); (2) protein synthesis measured by increase in protein nitrogen or activity of catalase and β-galactosidase; (3) nucleic acid synthesis. In no case has the removal of nucleic acid resulted in complete cessation of any of these processes but they have all been reduced by 98 % in 'good' experiments, and then been restored to the initial level by the addition of staphylococcal nucleic acid to the incubation mixture (except with β-galactosidase, where purines and pyrimidines are necessary, Gale & Folkes, 1955). The nucleic acid can be completely replaced by incorporation-factor preparation for the incorporation of a number of amino acids, and it is probable that other related factors are present in nucleic acid preparations, so that the whole activity of nucleic acid in this respect is attributable to such factors. The effect of nucleic acid on RNA synthesis can be replaced by the presence of incorporation factor and DNA. However, it has not been possible to demonstrate any effect of the factor on the development of catalase or β-galactosidase over and above its requirement for the synthesis of RNA or its presence in RNA. The number of enzymes whose synthesis can be studied satisfactorily in the disrupted staphylococcal cell is small, and the results obtained with catalase and β-galactosidase development may not be typical of the processes involved in protein synthesis in general. Nevertheless it is clear that the factor does not replace nucleic acid in these syntheses nor does it enhance the activity of suboptimum amounts of nucleic acid in promoting catalase development.

The requirement for amino acids in nucleic acid synthesis has led to the suggestion that proteins and nucleic acids arise from common intermediates (Ycas & Brawerman, 1957). However, the syntheses of protein and nucleic acid can be dissociated by the presence of chloramphenicol (Gale & Folkes, 1953*b*; Gros & Gros, 1958) or 8-azaguanine (H. Chantrenne, in preparation; M. H. Richmond, in preparation), and it may be that they are in fact separate processes. In that case, the incorporation factor may be more concerned with nucleic acid synthesis and the role of amino acids in that synthesis than in protein synthesis itself.

SUMMARY

1. A constant rate of incorporation of [^{14}C]-adenine into the nucleic acid fraction occurs when disrupted staphylococcal cells are incubated in the presence of adenosine triphosphate, hexose diphosphate and a mixture of all the amino acids required for protein synthesis, ribose, guanine, thymine and uracil. The incorporation can be correlated with an increase in the nucleic acid content of the preparation. Approximately 12% of the incorporated adenine is found in the deoxyribonucleic acid fraction.

2. Removal of nucleic acid from the disrupted cells results in a decrease of up to 98 % in the rate of adenine incorporation; the rate can be restored by addition of nucleic acid to the incubation mixture.

3. Nucleic acid can be replaced by the incorporation-factor preparation (Gale & Folkes, 1958); replacement is only complete if the full mixture of amino acids is present.

4. In disrupted cells highly depleted of nucleic acid, the incorporation factor is unable to replace nucleic acid completely if chloramphenicol is also present in the medium.

5. Under the experimental conditions used, chloramphenicol reduces the synthesis of deoxyribonucleic acid.

6. Deoxyribonucleic acid increases the activity of incorporation factor in promoting the incorporation of adenine into ribonucleic acid.

7. A number of inhibitors, including 8-hydroxyquinoline and derivatives of benzimidazole, inhibit the incorporation of adenine or guanine to the same extent as they inhibit the incorporation of glycine.

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Electrophoretic Behaviour of Human Serum Albumin at pH 4

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The separation of more than one moving boundary during electrophoresis of horse and human serum albumins in solutions of low ionic strength near pH 4, when first observed by Luetscher (1939), was assumed to be due to a 'true' heterogeneity of the albumins, i.e. to the existence of protein molecules differing intrinsically in electrochemical properties. Saifer & Corey (1954, 1955, 1956) were inclined to favour this view after obtaining multiple peaks with several serum albumins, ovalbumin and γ-globulin in buffer solutions of low ionic strength. On the other hand, Aoki & Foster (1957*a*, *b*) interpreted the results of their electrophoretic experiments with bovine serum albumin at low pH, as well as those of Phelps & Cann (1956), in terms of certain equilibria prevailing in the solutions. Since it is of considerable interest to establish whether or not the serum albumins exhibit a 'true' electrophoretic heterogeneity, several lines of approach have been taken in the present work.

A study has been made of the variations produced in the electrophoretic pattern of human serum albumin by various organic-buffer anions at constant pH, ionic strength and protein concentration. In some cases material isolated from the fastest-moving layers has been run again after further dialysis. Experiments have also been made with albumin labelled with radioactive iodine, and comparing specific activity and schlieren curves. The evidence accumulated does not support the case for 'true' electrophoretic heterogeneity, but favours the equilibrium hypothesis, a conclusion similar to that reached recently by Phelps & Cann (1957) in connexion with the electrophoresis of bovine serum albumin.

EXPERIMENTAL

Protein. The protein used in this work was the human serum albumin used previously (Charlwood, 1954), and distinguished by the designation A. For most experiments, solutions of rather more than 2% protein were dialysed against changes of buffer for several days. After the refractive-index increment had been measured at 578 mμ by means of a dipping refractometer, the protein solution was diluted to 2·00% (w/v), assuming a specific refractive increment of 0·00184 (Charlwood, 1957).

Buffers. These were mainly of ionic strength 0·02. The pH was adjusted to 4·00$\pm$0·05, as measured at room temperature (20°) with a Marconi instrument equipped with a glass electrode, and referred to 0·05 M-potassium hydrogen phthalate as standard (pH 4·00).

Electrophoresis experiments. These were done in the Tiselius apparatus (mostly at 2°), with the diagonal-schlieren optical system with a mercury-vapour lamp from which light of wavelength 546 mμ was isolated. The current used was usually not more than 4 mA in the standard cell, corresponding to a maximum voltage gradient of about 6·7 v/cm. in the buffer. Lower currents were essential in the runs at 20° (Johnson & Shooter, 1948). Only ascending boundaries were recorded, as the descending ones were very diffuse and anomalous under the conditions used. When extended runs were required, a photoelectric device was

used to control the position of the leading peak (Warren & Charlwood, 1953). As the faster-moving material isolated from a single run was insufficient for a subsequent run, the product of three similar runs was combined and concentrated by pressure dialysis before being used in the micro (3 ml.) cell.

Radioactive studies. A typical experiment with radioactive material was carried out as follows. A sample of the albumin was dissolved in buffer (1·28% glycine, 0·18% sodium hydroxide, 0·25% sodium chloride; pH 9·3) and labelled with ^{131}I by the jet method (McFarlane, 1956) to the extent of about one atom of total iodine/molecule, and a specific activity of 5 mc/g. A portion of iodinated protein was mixed with several times its weight of untreated albumin dissolved in lactate buffer, and thoroughly dialysed against this buffer. It was submitted to electrophoresis in the standard cell at a concentration of about 2% albumin, as in Fig. 1 (*c*), but for a longer time (about 3 hr.). The contents of the section containing the faster boundaries were isolated, mixed with more untreated albumin to bring the total protein concentration back to about 2%, and further dialysed. The next stage was a run in the flow-through type of cell (Kekwick, Lyttleton, Brewer & Dreblow, 1951), under the same conditions, and for about 2½ hr. The column of solution, slowly expressed from the exit capillary, was collected as 3 drop fractions (~0·15 ml.). These were each diluted with 3 ml. of buffer (or water with oxalate solutions), relative activities determined in a scintillation counter and protein concentrations measured in a Beckman DU spectrophotometer at 280 mμ wavelength. At this wavelength buffer absorption (except for oxalate of ionic strength 0·02) was negligible. Some runs with iodinated albumin were done in the flow-through cell without any preliminary separation of the faster layers. Specific-activity curves were correlated with the schlieren patterns by integrating the latter to different ordinates and comparing with the concentration–height curves constructed from the optical-density figures.

Sedimentation experiments. Solutions containing 1·5% of albumin in buffers of ionic strength 0·02 (pH 4) were examined in a Spinco ultracentrifuge operated under the usual conditions (Charlwood, 1954). Acetate, lactate and oxalate were used in these experiments.

RESULTS

The different types of pattern given in the presence of the various anions are illustrated in Fig. 1. Patterns for mandelate and salicylate are not included as they seemed to give rise to convections or precipitation in the cell. Whereas formate and barbiturate gave patterns of the lactate type, propionate, malate and succinate gave rise to the acetate type. In most of the buffers (Fig. 1) the leading peak was followed by a smaller one, which was absent in phthalate. This peak was also not visible in citrate if the pH were slightly higher (4·10). A third moving peak, also travelling towards the cathode, was resolved in lactate, formate and barbiturate (and also detected in citrate), and in citrate, oxalate and phthalate there was an additional boundary moving towards the anode. The boundaries which remained approximately stationary were probably of the δ type (Tiselius & Kabat, 1939). Continuation of the runs produced generally some further resolution, e.g. a slow-moving peak separating from the stationary one in acetate, and further subdivision of the leading peak in acetate, citrate, etc. (Fig. 2). These effects,

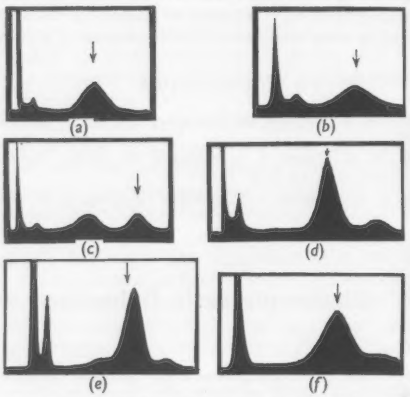

Fig. 1. Electrophoretic patterns in various buffers ($I=0·02$) at protein concentration of 2·00% (w/v). (Approximate positions of initial boundaries are indicated by arrows.) Temperature 2·0°, except in (*b*). (*a*) Acetate (pH 4·02), 60 min. at 6·5 v/cm.; (*b*) acetate (pH 4·04), 163 min. at 1·36 v/cm.; and 20·0°; (*c*) lactate (pH 3·97), 74 min. at 6·7 v/cm.; (*d*) oxalate (pH 3·99), 139 min. at 4·2 v/cm.; (*e*) citrate (pH 4·02), 86 min. at 6·3 v/cm.; (*f*) phthalate (pH 4·00), 279 min. at 5·0 v/cm. Patterns similar to (*a*) were given with propionate, malate and succinate, similar to (*c*) with formate and barbiturate.

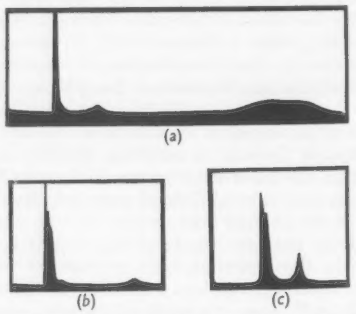

Fig. 2. Electrophoretic patterns ($I=0·02$) after further electrophoresis. Protein concentration, 2·00% (w/v). Migration was to the left. Temperature 2·0°. (*a*) Acetate (pH 4·02), 180 min. at 6·5 v/cm.; (*b*) acetate (pH 4·02), 300 min. at 6·5 v/cm.; (*c*) citrate (pH 4·02), 271 min. at 6·3 v/cm. [Slow-moving peaks are no longer visible in (*b*) and (*c*).]

which were reproducible, did not appear to be associated with convections.

Incorporation of 0·02 M-sodium chloride in the acetate and lactate buffers did not produce any marked effects on the pattern (Fig. 3*a*, *c*), but at the same total ionic strength (i.e. 0·04) and different proportions of acetate and chloride (0·036 M-sodium chloride, acetate of ionic strength 0·004) an extra moving peak occurred (Fig. 3*b*).

Variation of the protein concentration produced some alteration in the relative area distribution under the peaks, especially a reduction in size of the stationary boundary (Figs. 1*c*, 5*a*), but there was no change in the number of peaks (cf. Saifer & Corey, 1955).

The effect of using a temperature of 20° instead of 2° was to give rather more diffuse patterns, as might be expected, but otherwise little difference could be observed (e.g. Fig. 1*a*, *b*).

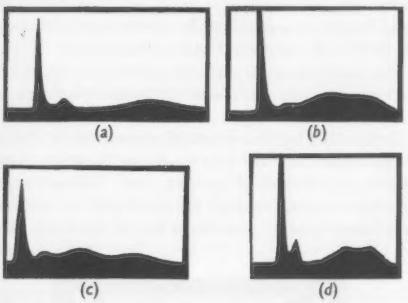

Fig. 3. Electrophoretic patterns at different buffer concentrations and protein concentration of 2·00% (w/v). Migration was to the left. Temperature 2·0°. (*a*) Acetate ($I = 0·02$) + chloride ($I = 0·02$), pH 4·00, 459 min. at 1·70 v/cm.; (*b*) acetate ($I = 0·004$) + chloride ($I = 0·036$), pH 3·95, 278 min. at 1·76 v/cm.; (*c*) lactate ($I = 0·02$) + chloride ($I = 0·02$), pH 4·03, 477 min. at 1·40 v/cm.; (*d*) lactate ($I = 0·04$), pH 4·00, 139 min. at 3·7 v/cm.

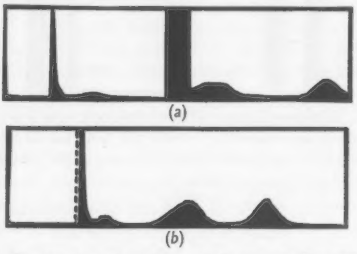

Fig. 4. Electrophoretic patterns in lactate buffer (pH 3·97, $I = 0·02$) after (*a*) 171 min. at 6·7 v/cm. and (*b*) 153 min. at 5·0 v/cm. Migration was to the left. Dotted line indicates the level of exit capillary in (*b*). Protein concentration, 2·0% (w/v). Temperature 2·0°.

The course of a typical re-running experiment is illustrated in Figs. 4 (*a*) and 5 (*b*). Material separated from the left-hand section in runs carried to the stage shown in Fig. 4 (*a*), after concentration by pressure dialysis, gave the pattern shown in Fig. 5 (*b*). The corresponding pattern obtained with unfractionated albumin (Fig. 5*c*) was indistinguishable from it.

The stages at which the faster layers were separated, and the radioactive fractions run off, are illustrated in Fig. 4 (*a*), (*b*). Curves obtained from runs in phosphate and lactate buffer in flow-through experiments are shown in Fig. 6. Curves of optical density (not shown) were consistent with ones derived from the schlieren curves by integration, but the former were rather flatter, showing that there was a small amount of mixing during the passage of the column of solution through the exit tubes from the cell. The specific activity curve in phosphate buffer (Fig. 6*a*) was linear, but in lactate it was composite, corresponding with the more complex refractive-index gradient curve. It is important, however, that the curves in Fig. 6 (*b*), (*c*) are identical in type, indicating that some of the protein which moved in the leading peaks in the first experiment had been converted into a form of lower mobility, whether by ion binding, change in molecular configuration or some other process.

In the ultracentrifuge runs only a single peak was observed (Fig. 7). Although it had some

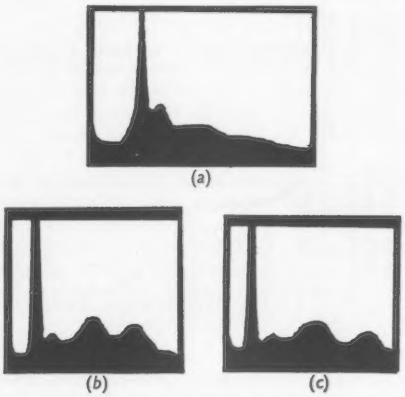

Fig. 5. Electrophoretic patterns in lactate ($I = 0·02$, pH 3·96). Migration was to the left. Temperature 2·0°. (*a*) 0·5% (w/v) protein after 150 min. at 3·4 v/cm.; (*b*) 1·35% (w/v) protein after 91 min. at 4·2 v/cm. in the micro-cell. Re-dialysed from isolated leading layers. (*c*) 1·36% (w/v) protein after 103 min. at 4·2 v/cm. in the micro-cell.

asymmetry, which might be expected under the conditions used, there was no evidence for the existence of aggregates.

DISCUSSION

Substitution of chloride for part of the buffer anion (Phelps & Cann, 1956) in these systems has the disadvantage of increasing the complexity of the system, and simultaneously altering the buffer capacity. It was hoped that, by the use of a variety of organic acids and their sodium salts, it would be possible to examine systems of comparable buffer capacity in which the main effects were clearly attributable to the specific ion effects alone. However, approximate calculation showed that there were considerable individual variations in buffer capacity (Table 1) when pH and ionic strength were standardized.

A comparison of Table 1 with Fig. 1 shows that, for the series of univalent buffers, the complexity of the electrophoretic pattern increased as buffer capacity decreased. A similar tendency was observed among the aliphatic dibasic acids. These results may be compared with the effect of chloride on patterns obtained in acetate. Where the buffer capacity was undiminished (Fig. 3*a*) the pattern was slightly more diffuse, but when the buffer capacity was reduced (Fig. 3*b*) an extra peak appeared. If the buffer capacity, relative to the concentration of protein present, were of great significance, a reduction of protein concentration (or an increase in buffer concentration) might alter the number of peaks obtained. In lactate buffer, at least, this did not occur (Figs. 3*d*, 5*a*). The actual differences of pH between various layers, even under conditions similar to those used here, have been found experimentally to be relatively small (E. F. Woods, personal communication).

The unfavourable ratio of salt to protein concentration is likely to result in large superimposed gradients of salt or protein at the boundaries. This is illustrated by the marked reduction in the size of the stationary boundary as the protein concentration is lowered (Figs. 1*c*, 5*a*). Measurements of relative peak areas or attempts to estimate mobilities would therefore be of limited value,

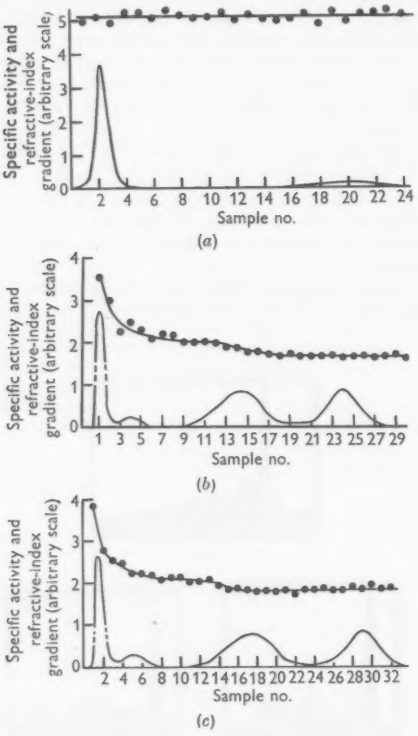

Fig. 6. Specific-activity (●) and refractive-index gradient curves for iodinated albumin in (*a*) phosphate ($I=0{\cdot}2$, pH 7·96) after 341 min. at 3·35 v/cm.; (*b*) lactate ($I=0{\cdot}02$, pH 4·00) after 153 min. at 5·0 v/cm.; (*c*) lactate ($I=0{\cdot}02$, pH 3·97) after 145 min. at 5·0 v/cm. In (*c*) the protein consisted of leading layers separated by preliminary electrophoresis of iodinated albumin, together with untreated carrier albumin. Temperature 2° in all cases.

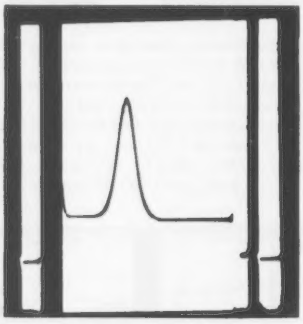

Fig. 7. Ultracentrifuge pattern obtained with 1·5% (w/v) albumin in oxalate ($I=0{\cdot}02$, pH 4·00) at 21·2°. Sedimentation (right to left) for 131 min. at 59 780 rev./min.

Table 1. *Relative buffer capacities at ionic strength* 0·02, pH 4·0

Propionate	1·00	Succinate	0·68
Acetate	0·95	Malate	0·46
Barbiturate	0·56	Oxalate	0·15
Lactate	0·47	Phthalate	0·18
Formate	0·38	Citrate	0·25

unless a reliable extrapolation to zero protein concentration could be made.

The effects observed, whether or not partly dependent on buffer capacity, must involve specific interaction between buffer anions and protein. This could involve albumin molecules with inherently different characteristics, or alternatively might arise from a population normally homogeneous, if binding of anions involves a statistical distribution (Klotz, 1953). In all the solutions the number of univalent buffer ions present is large (~ 50/albumin molecule) but bivalent or tervalent ions are present in much smaller numbers. A dynamic equilibrium, even if rapidly attained, might still result in the separation of distinct peaks, as has been demonstrated theoretically in a formally similar case in the ultracentrifuge (Gilbert & Jenkins, 1956). These authors considered an instantaneous equilibrium of the type $A+B \rightleftharpoons C$, where the three species have different sedimentation coefficients. Diffusion was neglected to enable the differential equations to be solved analytically, and curves were computed to show that more than one maximum could still arise in the schlieren pattern. Gilbert & Jenkins (1956) stated that, according to their theory, the boundary keeps its essential shape, and merely stretches out with time. If equilibrium were not attained instantaneously, we should expect the characteristics of the pattern to be intermediate between this and the normal type given by three species sedimenting independently. The complexity of the schlieren pattern could conceivably increase with time if the equilibrium required a moderate time to be established. The splitting of the leading peak at longer times in the present experiments may be such an example. The minor effects of temperature alterations are not incompatible with ion-binding, which is often not greatly affected by differences of temperature (Klotz, 1953). The more strongly an anion is bound at pH 4, the lower becomes the net charge (and most probably the mobility) of the protein. Very strong binding might produce a reversal of the sign of the charge when the protein is not too far from the isoelectric point. Of the three buffers in which a boundary was detected moving towards the cathode, one at least (phthalate) is known to bind strongly to albumin (Klotz & Urquhart, 1949). The slow rate of movement of the main peak in phthalate, compared with most of the other buffers of ionic strength 0·02, would also be consistent with strong binding. The leading boundary, and the smaller one observed adjacent to it in most buffers, may correspond with the two interconvertible forms of albumin postulated by Aoki & Foster (1957*a*, *b*). This is supported by the disappearance of the smaller one in citrate when the pH is slightly higher.

The re-running experiments clearly demonstrated that the material which migrated in the leading boundaries was redistributed after dialysis. In contrast with this, Saifer & Corey (1956) claimed that the fastest layer separated from human γ-globulin could be re-run as a single peak, although their second run was done at a much lower concentration.

Iodinated albumin helped to confirm the conclusions drawn from the re-running experiments, but the specific-activity curves were not susceptible of a simple interpretation. The sharp rise of specific activity through the two leading peaks in Fig. 6 (*b*), (*c*) is probably due to an increase in electrophoretic mobility of albumin molecules when iodine substituted, a suggestion supported by other evidence (Charlwood & Gordon, 1958). The variation in specific activity through the third peak may be due to a similar mobility effect, but it could also be due, partly or wholly, to different anion-binding characteristics of substituted and non-substituted albumin molecules. The plateau region of the activity curve corresponding with the stationary boundary supports the contention that this boundary represents nearly a pure dilution effect (δ boundary).

In earlier work (Charlwood, 1954) no differences were detectable in the diffusion and sedimentation behaviour of whole albumin and that in the leading boundary in acetate buffer, ionic strength 0·02, pH 4·0. This is consistent with the ultracentrifugal evidence presented here against the hypothesis that aggregation might be a contributing factor.

It is concluded that the behaviour of human albumin at pH 4 does not afford any evidence of a 'true' electrophoretic heterogeneity, although it does not prove that albumin does not contain families of molecules differing fundamentally in some other physical properties (cf. the solubility studies of Derrien, 1947). The observed patterns must be produced by anion interaction, which may also account for the apparent heterogeneity of human albumin on prolonged running in phosphate (Blix, Tiselius & Svensson, 1941; Hoch & Morris, 1945) and in veronal buffer (Charlwood, 1954).

SUMMARY

1. Electrophoretic patterns have been obtained for human albumin at pH 4 and low ionic strength (0·02) in the presence of various organic anions.

2. The faster-moving layers in some instances were removed and run again after further dialysis.

3. Albumin labelled with radioactive iodine was used in experiments designed to show the variations of specific activity through the different moving boundaries.

4. It is concluded that all observations are to be explained in terms of protein–ion interactions rather than true protein heterogeneity.

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Paper Chromatography of Saturated and Unsaturated Fatty Acids

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During an investigation of fat metabolism in the fungus *Trichoderma viride* it became desirable, in order to avoid culturing excessive quantities of mycelium, to find a method of separating micro-quantities of natural fatty acids. Such a method would also be useful for radioautographic purposes. In this communication the behaviour of over 40 pure fatty acids on several reversed-phase paper-chromatographic systems is examined: the systems are liquid paraffin/aqueous acetone (adapted from a column-chromatographic method, see Howard & Martin, 1950; Silk & Hahn, 1954; Crombie, Comber & Boatman, 1955), liquid paraffin/aqueous acetic acid (a system resembling that of Kaufmann & Nitsch, 1954; Wagner, Abisch & Bernhard, 1955; Seher, 1956; Leibnitz *et al.* 1957), castor oil/aqueous acetone (adapted from the column system of Savary & Desnuelle, 1953; Matic, 1956), castor oil/aqueous acetic acid, and polythene/aqueous acetone (adapted from the column method of Green, Howitt & Preston, 1955).

METHODS AND MATERIALS

Impregnation of papers

(1) *Liquid paraffin.* Whatman no. 1 chromatography papers (13 cm. × 45 cm.) were immersed in a solution of liquid paraffin (Nujol) (35 ml.) in benzene (65 ml.), blotted (10 min.) under an evenly distributed 18 lb. weight and dried by hanging in air at room temperature. Provided that the same grade of blotting paper is used, a constant weight of paraffin (25%) remains on each paper. It is important to avoid excess of paraffin since this results in loss of fatty acid during formation of the copper salts (for full details see below under 'Quantitative paper chromatography of fatty acids'). After removal of the top (11 cm.) and bottom (4 cm.) portions, each paper was cut into standard 30 cm. × 2 cm. lengths ('top' and 'bottom' refers to a paper hung vertically with the direction of machining pointing upwards).

(2) *Castor oil.* A solution of castor oil (B.P. grade) in ethyl ether was shaken three times with 4% (w/v) aq. K_2CO_3 to remove free fatty acids. After the solution had been washed three times with water and dried (Na_2SO_4) and the solvent evaporated, the neutral oil (35 ml.) was dissolved in benzene (65 ml.) and this solution used for impregnating papers as in (1) above.

(3) *Polythene.* Polythene powder (Schori Metallising Process Ltd.) was dissolved in xylene [3 or 6% (w/v) final solution] at 60° and papers impregnated with the hot solution. They were air-dried at 20° until solvent-free.

Application of fatty acids to the paper

Pure fatty acids or mixtures, dissolved in $CHCl_3$ (approx. 6 mg./ml.), were delivered (1–5 μl. at a time, total volume 10–20 μl.) on the starting line (5 cm. from the upper edge

of the paper) by means of an Agla micrometer syringe. After evaporation of the solvent the papers were equilibrated (4 hr.) in contact with vapour from the eluting solvent, before downward development.

Elution systems

(1) *Paraffin/acetic acid.* Fatty acids on paraffin-impregnated papers were eluted with 90% (system 1A), 95% (1B), 70% (1C) or 50% (1D) (v/v) aq. acetic acid, previously equilibrated with paraffin as described by Wagner *et al.* (1955). Elution was continued at 21° (±0·5°) for 16 hr.: the solvent front travelled from 16–18 cm. (1A) to 23–26 cm. (1D). After the position of the solvent front had been marked the papers were dried by hanging in air.

(2) *Paraffin/acetone.* Fatty acids on paraffin-impregnated papers were eluted with 90% (v/v) aq. acetone which had been previously equilibrated with paraffin by shaking and standing for 24 hr. (system 2). After elution for 8 hr. the solvent front had advanced 22–23 cm.

(3) *Castor oil/acetic acid.* Fatty acids on castor-oil-impregnated papers were eluted with 70% (system 3A) or 50% (3B), v/v, aq. acetic acid, which had been equilibrated with castor oil by the technique of (1) above. After elution for 16 hr. the solvent front had advanced 9–14 cm. (3A) or 17–20 cm. (3B).

(4) *Castor oil/acetone.* Fatty acids on castor-oil-impregnated papers were eluted with 50% (v/v) aq. acetone (system 4), which had been equilibrated with castor oil as in (1) above. After elution for 10 hr. the solvent front had travelled 14–15 cm.

(5) *Polythene/acetone.* Fatty acids on polythene papers were eluted with 95, 90, 80, 70 or 60% (v/v) aq. acetone (system 5), which had been equilibrated with polythene by the technique of (2) above. Elution times varied from 4 hr. (95% acetone) to 10 hr. (60% acetone).

Detection of fatty acid spots

After the papers had been dried in air at room temperature, copper salts of the fatty acids were formed by immersing the papers in cupric acetate solution (20 ml. of a saturated solution in 1 l. of water) for 20 min. (Wagner *et al.* 1955). Excess of copper was removed by washing for 15 min. in dilute acid (0·2 ml. of acetic acid/l.). If quantitative results are not required a further 5–10 min. wash in fresh dilute acetic acid improved the differentiation of acid spots from the background, but causes loss of copper, particularly from acids of high R_F, e.g. linolenic acid or lauric acid. After drying, the copper was detected by immersing the papers (30 sec.) in 0·03% (w/v) dithio-oxamide (rubeanic acid) in ethanol. This method produces light-stable dark-green spots against a lighter background and can detect 0·1 μg. of copper (Vaeck, 1955). Excess of dithio-oxamide is removed by washing for 2 min. in ethanol.

Sources of fatty acids

Saturated *normal* even-numbered acids of chain length C_{12}–C_{20} were obtained from Professor N. K. Adam, F.R.S., the C_{10} acid from British Drug Houses Ltd. and the C_{22} acid from Hess Products Ltd. The C_{11} *normal* acid was obtained from Dr L. Crombie, *normal* odd-numbered acids (C_{13}–C_{19} inclusive) from Dr F. B. Shorland and the branched-chain C_{19} (tuberculostearic) acid from Dr B. C. L. Weedon. Oleic, linoleic and linolenic acids came from the California Foundation for Biochemical Research, U.S.A., *erythro-* and *threo*-dihydroxystearic acid, elaidic acid, petroselinic acid, tariric acid and erythrogenic acid from Dr B. C. L. Weedon, ximenynic acid from Dr S. P. Ligthelm, *cis-* and *trans*-octadec-2-enoic acid from Dr G. S. Myers and the remaining acids from Dr L. Crombie. Details on the purity of some of these specimens have already been recorded (Crombie *et al.* 1955), and each sample was tested for purity chromatographically. The melting points of the highly unstable conjugated octadecatrienoic acids were checked immediately before use and corresponded exactly with those reported for the pure acids (Crombie & Jacklin, 1957).

Spores of *Trichoderma viride* were obtained from cultures grown on agar slopes on a 20% glucose medium in the light. Separation was achieved by shaking each tube with water, pouring the suspension through a sieve (to remove fragments of mycelium) and centrifuging. Spore-free mycelium was grown in a 20% glucose liquid medium in the dark and separated from the culture fluid by filtration. Material was killed by immersion in boiling ethanol and the lipids were extracted with ethanol and diethyl ether. A crude separation into phosphatides and glycerides was effected with ice-cold acetone, and the fatty acids were extracted from each fraction by standard techniques (Hardman & Crombie, 1958).

RESULTS

Qualitative separation of fatty acids in different reversed-phase systems

Polythene/aqueous acetone (*system* 5). Satisfactory separation of *normal* even-numbered saturated acids of chain length C_{14}–C_{18} was achieved, although it proved impossible to prevent 'tailing'. Further development of this system was discontinued when it became apparent that it was less promising than others described below.

Paraffin/aqueous acetone (*system* 2). Separation of *normal* saturated acids (C_{14}–C_{20}) was achieved (Table 1), but the fatty acid spots were diffuse: the solvent front travels rapidly, which may account for the effect.

Paraffin/aqueous acetic acid (*system* 1 *A–D*). This system gave excellent separations of the *normal* even-numbered saturated fatty acids (C_{10}–C_{22}) (Table 1). Acids of medium chain length (C_{10}–C_{14}) separate best in more aqueous solvents (system 1C), and long-chain acids (C_{18}–C_{22}) in the less aqueous solvents (1B). The acids usually encountered in plant tissues (C_{14}–C_{20}) are most conveniently separated in 90% acetic acid (1A). These results agree with those of Kaufmann & Nitsch (1954), who used a similar system with paraffin of lower boiling point. Acids of chain length less than C_{10} cannot be detected by the copper/dithio-oxamide method owing to the solubility of their copper salts. R_F values are recorded in Table 1 as mean values, but, although these serve as a useful guide, the only decisive test of separability is chromatography of appropriate mixtures. Thus some of the acids with closely similar R_F values do in fact separate.

Table 1. *Separation of saturated fatty acids on different paper-chromatographic systems*

Systems: 1A, paraffin/90% acetic acid; 1B, paraffin/95% acetic acid; 1C, paraffin/70% acetic acid; 2, paraffin/90% acetone; 3A, castor oil/70% acetic acid.

Fatty acid	Structure	System	No. of chromato-grams	Mean R_F	Standard error
Capric	C_{10} *normal* chain	1C	2	0·57	0·020
Undecanoic	C_{11} *normal* chain	1C	10	0·45	0·029
Lauric	C_{12} *normal* chain	1C	4	0·33	0·087
		1A	12	0·53	0·014
Tridecanoic	C_{13} *normal* chain	1A	5	0·60	0·020
Myristic	C_{14} *normal* chain	1A	12	0·38	0·015
		2	2	0·69	0·000
		3A	2	0·29	0·015
Pentadecanoic	C_{15} *normal* chain	1A	5	0·42	0·022
Palmitic	C_{16} *normal* chain	1A	25	0·29	0·012
		2	2	0·58	0·005
		3A	2	0·12	0·010
Margaric	C_{17} *normal* chain	1A	5	0·27	0·019
Stearic	C_{18} *normal* chain	1A	43	0·16	0·007
		1B	3	0·34	0·018
		2	2	0·45	0·010
		3A	4	0·00	0·000
Nonadecanoic	C_{19} *normal* chain	1A	5	0·11	0·060
Tuberculostearic	$CH_3 \cdot [CH_2]_7 \cdot CHMe \cdot [CH_2]_8 \cdot CO_2H$	1A	4	0·12	0·008
Arachidic	C_{20} *normal* chain	1A	10	0·10	0·010
		2	2	0·33	0·010
Behenic	C_{22} *normal* chain	1A	3	0·06	0·004
		1B	1	0·14	—

The *normal* odd-numbered saturated acids (C_{11}–C_{19}) were completely separable, again confirming that a difference in chain length of $\cdot[CH_2]_2\cdot$ is sufficient for successful chromatography. When odd- and even-numbered acids are mixed, the success of separation depends on the chain length, e.g. C_{10}, C_{11}, C_{12}, C_{13}, C_{14} and C_{15} acids form discrete spots, but satisfactory separation of the C_{16}, C_{17}, C_{18} and C_{19} acids could not be achieved. Rather similar results have been obtained on radial chromatograms (Leibnitz *et al.* 1957). Branching of the acid chain, as in tuberculostearic acid, had little effect, this acid behaving similarly to the C_{19} *normal* acid. Such results are closely similar to those obtained with a paraffin/aqueous acetone column system (Howard & Martin, 1950; Silk & Hahn, 1954; Crombie *et al.* 1955; Garton & Lough, 1957).

Incorporation of an ethylenic linkage increases the R_F, but the degree of separation from a *normal* saturated acid of the same chain length is dependent on the position of unsaturation. Among the C_{18} monoethylenic acids the octadec-2-enoic acids (both *cis*- and *trans*-) are inseparable from octadecanoic (stearic) acid; octadec-6-enoic (petroselinic) acid separates moderately well and both *cis*- and *trans*-octadec-9-enoic acids (oleic and elaidic) are completely separable from stearic acid (Table 2). When the double bond is well removed from the carboxyl group the effect is to increase the R_F to such an extent that the acid is eluted with the *normal* saturated acid of chain length $\cdot[CH_2]_2\cdot$ less: e.g. erucic (C_{22} monoene) is eluted with arachidic (C_{20}) acid; petroselinic acid, oleic acid and elaidic acid (C_{18} monoene) are eluted with palmitic (C_{16}) acid. *cis*- and *trans*-Isomers (e.g. *cis*- and *trans*-octadec-2-enoic acids and *cis*- and *trans*-octadec-9-enoic acids) are not separable.

The R_F of a monoethenoid fatty acid is increased further by incorporating extra double bonds in the centre of the chain; e.g. linoleic (C_{18} diene) is eluted with myristic (C_{14}) acid and linolenic (C_{18} triene) acid is eluted with lauric (C_{12}) acid. Each additional ethylenic linkage increases the R_F to approximately the same extent as the removal of $\cdot[CH_2]_2\cdot$ from a saturated *normal* acid. Conjugated octadecatrienoic (α- and β-elaeostearic and -punicic) acids have similar R_F values to that of a non-conjugated octadecatrienoic (linolenic) acid, but the former are too unstable for accurate paper chromatography. When the normal procedure of equilibrating for 4 hr. before eluting was followed, these acids decomposed to gums which remained on the starting line. When they were applied to the papers immediately before elution, or chromatographed in an inert atmosphere, breakdown was considerably reduced, but there were still traces of

Table 2. *Separation of ethylenic and acetylenic acids on different paper-chromatographic systems*

See Table 1 for key to systems.

Fatty acid	Structure	System	No. of chromatograms	Mean R_F	Standard error
Monoethylenic					
Undec-10-enoic	$CH_2{=}CH{\cdot}[CH_2]_8{\cdot}CO_2H$	1C	6	0·57	0·024
Oleic	$CH_3{\cdot}[CH_2]_7{\cdot}CH^{c}{=}CH{\cdot}[CH_2]_7{\cdot}CO_2H$	1A	34	0·26	0·010
Elaidic	$CH_3{\cdot}[CH_2]_7{\cdot}CH^{t}{=}CH{\cdot}[CH_2]_7{\cdot}CO_2H$	1A	6	0·33	0·014
Petroselinic	$CH_3{\cdot}[CH_2]_{10}{\cdot}CH^{c}{=}CH{\cdot}[CH_2]_4{\cdot}CO_2H$	1A	8	0·21	0·014
Octadec-*cis*-2-enoic	$CH_3{\cdot}[CH_2]_{14}{\cdot}CH^{c}{=}CH{\cdot}CO_2H$	1A	4	0·20	0·003
Octadec-*trans*-2-enoic	$CH_3{\cdot}[CH_2]_{14}{\cdot}CH^{t}{=}CH{\cdot}CO_2H$	1A	5	0·19	0·006
Erucic	$CH_3{\cdot}[CH_2]_5{\cdot}CH^{c}{=}CH{\cdot}[CH_2]_{11}{\cdot}CO_2H$	1A	1	0·12	—
		1B	3	0·25	0·010
Diethylenic					
Linoleic	$CH_3{\cdot}[CH_2]_4{\cdot}CH^{c}{=}CH{\cdot}CH_2{\cdot}CH^{c}{=}CH{\cdot}[CH_2]_7{\cdot}CO_2H$	1A	31	0·40	0·013
		2	2	0·72	0·020
Triethylenic					
Linolenic	$CH_3{\cdot}CH_2{\cdot}CH^{c}{=}CH{\cdot}CH_2{\cdot}CH^{c}{=}CH{\cdot}CH_2{\cdot}CH^{c}{=}CH{\cdot}[CH_2]_7{\cdot}CO_2H$	1A	22	0·53	0·012
α-Elaeostearic	$CH_3{\cdot}[CH_2]_3{\cdot}CH^{t}{=}CH{\cdot}CH^{t}{=}CH{\cdot}CH^{c}{=}CH{\cdot}[CH_2]_7{\cdot}CO_2H$	1A	4	0·46	0·012
β-Elaeostearic	$CH_3{\cdot}[CH_2]_3{\cdot}CH^{t}{=}CH{\cdot}CH^{t}{=}CH{\cdot}CH^{t}{=}CH{\cdot}[CH_2]_7{\cdot}CO_2H$	1A	3	0·37	0·022
Punicic	$CH_3{\cdot}[CH_2]_3{\cdot}CH^{c}{=}CH{\cdot}CH^{t}{=}CH{\cdot}CH^{c}{=}CH{\cdot}[CH_2]_7{\cdot}CO_2H$	1A	4	0·46	0·002
Acetylenic					
Undec-10-ynoic	$CH{\equiv}C{\cdot}[CH_2]_8{\cdot}CO_2H$	1C	6	0·76	0·013
Stearolic	$CH_3{\cdot}[CH_2]_7{\cdot}C{\equiv}C{\cdot}[CH_2]_7{\cdot}CO_2H$	1A	12	0·45	0·011
Tariric	$CH_3{\cdot}[CH_2]_{10}{\cdot}C{\equiv}C{\cdot}[CH_2]_4{\cdot}CO_2H$	1A	6	0·40	0·005
Ximenynic	$CH_3{\cdot}[CH_2]_5{\cdot}CH{=}CH{\cdot}C{\equiv}C{\cdot}[CH_2]_3{\cdot}CO_2H$	1A	4	0·59	0·005
Erythrogenic	$CH_2{=}CH{\cdot}[CH_2]_4{\cdot}C{\equiv}C{\cdot}C{\equiv}C{\cdot}[CH_2]_7{\cdot}CO_2H$	1A	5	0·71	0·034
		1C	4	0·47	0·015
Octadec-11-ene-9:13-diynoic	$CH_3{\cdot}[CH_2]_3{\cdot}C{\equiv}C{\cdot}CH{=}CH{\cdot}C{\equiv}C{\cdot}[CH_2]_7{\cdot}CO_2H$	1A	2	0·51	0·015
		1C	3	0·35	0·021

gum on the starting line and traces of another acid of higher R_F, presumably another breakdown product. Such decomposition of these very unstable acids is avoided by column chromatography (Crombie *et al.* 1955). Difficulty is likely to be considerable in other paper-chromatographic methods which involve heating to remove paraffin from the papers.

The introduction of an acetylene linkage has a marked effect on R_F; octadec-9-ynoic (stearolic) acid and octadec-6-ynoic (tariric) acid are completely separable from the corresponding octadecanoic acids (oleic acid and petroselinic acid), the R_F being increased to approximately the same extent as by the addition of two ethylenic linkages or the removal of $\cdot[CH_2]_4\cdot$ from a saturated chain (Table 2, Fig. 1). An extra ethylenic linkage, conjugated with the acetylene linkage as in ximenynic acid, increases the R_F still further and makes possible a separation from the acetylenic acid. Extra acetylenic linkages also increase the R_F (e.g. erythrogenic acid and octadec-11-ene-9:13-diynoic acid), but the effect of two conjugated acetylenic linkages with the ethylenic linkage in the ω-position (as in erythrogenic acid) is greater than that of an yn-en-yn conjugated system and the two acids are completely separable.

The presence of a hydroxyl group increases the R_F considerably (Table 3); e.g. both 8- and 12-hydroxystearic acids are completely separable from stearic acid, ricinoleic acid and ricinstearolic acid from oleic acid and stearolic acid, and α-kamlolenic acid from α-elaeostearic acid. When two hydroxyl groups are present, as in the dihydroxystearic acids, there is no separation from monohydroxystearic acids.

An oxo group also increases the R_F but to a somewhat smaller extent than a hydroxyl group, e.g. 12-hydroxystearic acid and 12-oxostearic acid are partially separable in 90% acetic acid. The presence of both an oxo and a hydroxy group (e.g. 12-hydroxy-10-oxostearic acid or 12-hydroxy-9-oxostearic acid) increases the R_F still further.

Castor oil/aqueous acetic acid (systems 3A and B) and castor oil/aqueous acetone (system 4). Since mono- and di-hydroxystearic acid were inseparable on paraffin systems, two castor-oil systems were investigated for this purpose. The more successful was castor oil/aqueous acetic acid (systems 3A and 3B, Tables 1 and 3), and by using this good separations of mono- and di-hydroxystearic acid were achieved. This system should be of value for separating cuticle hydroxy acids and for separating the hydroxylation products of mixed unsaturated acids. For mixtures containing only monohydroxy acids, however, the paraffin system is preferred, since this can be used with eluting solvents containing up to 95% of acetic acid, whereas castor-oil papers are limited to more aqueous mixtures because of the solubility of castor oil in aqueous acetic acid stronger than 75% (v/v).

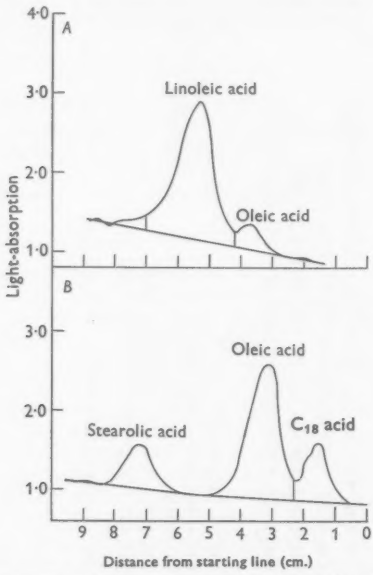

Fig. 1*A*. Use of paper chromatography to indicate acid purity. The sample is a commercial sample of linoleic acid (not from the California Foundation for Biochemical Research). Analysis by paper chromatography shows linoleic acid (88%), oleic acid (12%); figures from column chromatography (Crombie *et al.* 1955) were 87·5 and 12·5%. Linoleic acid content determined by ultraviolet spectrophotometry (Brice, Swain, Herb, Nichols & Riemenschneider, 1952) was 86%. Similar results for a commercial sample of linoleic acid were obtained by Wagner *et al.* 1955. *B*, Separation of stearolic acid, oleic acid and stearic acid. Molar percentage composition (weighed), stearolic acid 20, oleic acid 60, stearic acid 20; molar percentage composition (determined by paper chromatography), stearolic acid 19, oleic acid 62, stearic acid 19.

Quantitative paper chromatography of fatty acids

Standard mixtures. Quantitative methods for estimating micro amounts of fatty acids on either paraffin- or silicone-impregnated paper have been described (Wagner *et al.* 1955; Perila, 1956; Seher, 1956; Gellerman & Schlenk, 1956; Schlenk, Gellerman, Tillotson & Mangold, 1957; Bernhard, Abisch & Wagner, 1957; Leibnitz *et al.* 1957). Direct photometric measurement of the light-absorption of suitable derivatives on the paper is the method generally used, and indicators are of

Table 3. *Separation of hydroxy and oxo acids on different paper-chromatographic systems*

See Table 1 for systems: 1 D, paraffin/50% acetic acid; 3 B, castor oil/50% acetic acid; 4, castor oil/50% acetone.

Fatty acid	Structure	System	No. of chromatograms	Mean R_F	Standard error
8-Hydroxystearic	$CH_3 \cdot [CH_2]_5 \cdot CH(OH) \cdot [CH_2]_6 \cdot CO_2H$	1A	4	0·77	0·031
12-Hydroxystearic	$CH_3 \cdot [CH_2]_5 \cdot CH(OH) \cdot [CH_2]_{10} \cdot CO_2H$	1A	3	0·88	0·003
		1C	2	0·74	0·000
		3A	6	0·15	0·002
threo-9:10-Dihydroxystearic	$CH_3 \cdot [CH_2]_7 \cdot CH(OH) \cdot CH(OH) \cdot [CH_2]_7 \cdot CO_2H$	1A	1	0·89	—
		1C	2	0·87	0·005
		3A	6	0·38	0·011
		4	1	0·05	—
erythro-9:10-Dihydroxystearic	$CH_3 \cdot [CH_2]_7 \cdot CH(OH) \cdot CH(OH) \cdot [CH_2]_7 \cdot CO_2H$	1A	2	0·91	0·002
		1C	1	0·91	—
		3A	6	0·38	0·013
Ricinoleic	$CH_3 \cdot [CH_2]_5 \cdot CH(OH) \cdot CH_2 \cdot CH{=}CH \cdot [CH_2]_7 \cdot CO_2H$	1A	2	0·83	0·005
		1C	1	0·79	—
		1D	4	0·55	0·015
		3A	2	0·78	0·000
		3B	2	0·03	0·005
		4	1	0·03	—
α-Kamlolenic	$CH_2OH \cdot [CH_2]_{13} \cdot CH{=}CH \cdot CH{=}CH \cdot CH{=}CH \cdot [CH_2]_7 \cdot CO_2H$	1A	2	0·93	0·026
		1C	2	0·97	0·016
12-Hydroxydodec-10-ynoic	$CH_2OH \cdot C{\equiv}C \cdot [CH_2]_8 \cdot CO_2H$	1A		Soluble copper soap	
Ricinstearolic	$CH_3 \cdot [CH_2]_5 \cdot CH(OH) \cdot CH_2 \cdot C{\equiv}C \cdot [CH_2]_7 \cdot CO_2H$	1A	2	0·83	0·005
		1C	1	0·72	—
		1D	3	0·00	0·000
		3B	2	0·00	0·000
		4	1	0·04	—
Homoricinstearolic	$CH_3 \cdot [CH_2]_4 \cdot CH(OH) \cdot CH_2 \cdot C{\equiv}C \cdot [CH_2]_8 \cdot CO_2H$	1C	3	0·83	0·015
Oxo acids					
12-Oxostearic	$CH_3 \cdot [CH_2]_5 \cdot CO \cdot [CH_2]_{10} \cdot CO_2H$	1A	4	0·81	0·010
		3A	2	0·63	0·005
12-Hydroxy-9-oxostearic	$CH_3 \cdot [CH_2]_5 \cdot CH(OH) \cdot [CH_2]_6 \cdot CO \cdot [CH_2]_7 \cdot CO_2H$	1C	1	0·87	—
12-Hydroxy-10-oxostearic	$CH_3 \cdot [CH_2]_5 \cdot CH(OH) \cdot CH_2 \cdot CO \cdot [CH_2]_8 \cdot CO_2H$	1C	1	0·89	—
		3A	2	0·87	0·020

various types, e.g. iodine vapour and α-cyclodextrin (Schlenk *et al.* 1957), silver salts (Perila, 1956), mercuric acetate derivatives (Inouye, Noda & Hirayama, 1955), or estimation of the copper present in the copper salts after conversion into copper ferrocyanide (Wagner *et al.* 1955; Seher, 1956; Leibnitz *et al.* 1957).

The green complex formed by the reaction of copper soaps with dithio-oxamide, which is used to detect fatty acids in the present work, is also suitable for quantitative assay of copper by direct photometry (Vaeck, 1955; Lacourt & Hendryckx, 1956), but has not previously been applied to the quantitative determination of copper in the copper soaps of fatty acids. Photometric measurements were made by advancing paper strips past the photoelectric cell in a Scanner (Evans Electroselenium Ltd., red filter) and determining light-absorption at 1 mm. intervals. The slit dimensions were 1 mm. × 17 mm. and translucency was improved by soaking the papers for 2 hr. in liquid paraffin (Takahara & Taniguchi, 1955). The areas of the light-absorption peaks obtained were measured with a planimeter. A rather high but regular background line, horizontal or slightly sloping, was normally obtained. Peak areas were estimated by dropping verticals to this line from the lowest points between peaks (Fig. 1) as recommended by Crook, Harris, Hassan & Warren, 1954; Vaeck, 1955.

By using standard cupric sulphate solution it was confirmed that 0·1 μg. of copper was detectable and that peak area is proportional to the weight of copper in the range 0·1–7·0 μg. (corresponding to approximately 1–63 μg. of fatty acid/spot). Seher (1956) showed that fatty acids, both saturated and containing ethylenic linkages, react stoicheiometrically with cupric acetate and, in the present experiments, similar results were obtained with fatty acids containing one or more non-terminal acetylenic linkages. The method is therefore applicable to a wide range of unsaturated fatty acids. It is not suitable for quantitative work on acids of chain length below C_{12} owing to the solubility of their copper soaps.

When paraffin is present on the paper the amount of copper fixed per weight of fatty acid is less than the theoretical. We attribute this effect to 'lifting' of fatty acid in the paraffin layer when the papers are immersed in cupric acetate solution. Acids of low R_F, i.e. those preferentially soluble in the paraffin phase, retain less copper than those of high R_F (Table 4). Careful control of the weight of paraffin in the paper reduces this effect, but for quantitative work additional accuracy was obtained by correction from a standard mixture of approximately the same composition as the unknown sample. This was chromatographed on an adjacent paper strip on the same occasion. Schlenk *et al.* (1957) also had to resort to this expedient in their quantitative work on silicone-impregnated papers. At least three replicates of each mixture were used.

Analysis of natural mixtures of fatty acids. Fatty acids from natural sources contain both saturated and unsaturated components and, as shown above, these are not all separable chromatographically, e.g. palmitic acid and oleic acid have similar R_F values and so have linoleic acid, palmitoleic acid and myristic acid. From a chromatogram of the original mixture, however, the total quantity of acid in each peak can be estimated. Several methods were considered for the analysis of these mixed peaks. Formation and separation of lead salts into ethanol- or acetone-soluble (unsaturated) and -insoluble (saturated) fractions, followed by chromatography of the two fractions, has been used by Wagner *et al.* (1955). However, complete separation is difficult to achieve in the lead-salt method and, further, unsaturated acids of similar R_F values are not separable. Halogenation with Hanus iodine (Kobrle & Zahradnik, 1954) proved unsuitable for use with paraffin-impregnated papers, since the R_F values of halogenated acids were similar to those of the original unsaturated acids.

Hydrogenation (in acetic acid over Adams platinum oxide catalyst), followed by chromatography, was finally used to determine the total amount of acid of each chain length. If only one unsaturated component is present in each peak, direct computation from the results of the original and hydrogenated chromatograms can be used to determine acid composition. Results for the analysis, by this method, of two fatty acid mixtures from *Trichoderma viride* are shown in Table 5.

Table 4. *Quantitative analysis of standard mixtures without correction*

Plain figures, molar % (found); figures in parentheses, molar % (theoretical). Each figure is the mean of three replicates.

	Linolenic acid	Linoleic acid	Oleic acid + C_{16}		C_{18}	C_{22}
Mixture 1	—	49 (48)	51 (52)	—	—	—
2	7 (7)	36 (39)	44 (46)	—	10 (6)	3 (2)
3	—	—	—	23 (16)	77 (84)	—
4	—	—	—	20 (9)	76 (87)	—

Table 5. *Quantitative analysis of* Trichoderma viride *fatty acids by comparison of composition before and after hydrogenation*

u, Uncorrected molar %; c, corrected molar %; d, molar % analysis by column chromatography (Crombie *et al.* 1955).

		Linolenic acid	Linoleic acid	Oleic acid	C_{16}	C_{18}	C_{22}
Spore phosphatide acids	(u)	—	50	29	21	—	—
	(c)	—	49	36	15	—	—
	(d)	—	48	36	16	—	—
Mycelium glyceride acids	(u)	7	35	25	17	13	3
	(c)	8	37	37	8	8	2
	(d)	7	39	35	11	6	2

Correction from standard mixtures was applied both to the original chromatogram and to that of the hydrogenated acids: it is particularly important with the latter, which contains a high proportion of stearic acid. The results are compared with an analysis by the well-tested method of reversed-phase column chromatography (Crombie *et al.* 1955) and show good agreement.

If there is more than one unsaturated component in an acid peak, a further set of data can be obtained by destroying the unsaturated components by Bertram oxidation (strong alkaline permanganate at room temperature for 16–18 hr.) and chromatographing the remaining saturated fatty acids (the plant acids usually encountered have degradation products of high R_F which are completely separable from acids of chain length above C_{12}). Comparison of such a chromatogram with an original one and one after hydrogenation will indicate if there is more than one unsaturated acid in any mixed peak, and appropriate computation can be made.

The method is obviously not suitable for the analysis of highly complex mixtures such as those of fish oils, nor for those containing appreciable amounts of short-chain acids such as palm-kernel oil. It is, however, applicable to a wide range of natural plant fatty acids and has been used successfully with fats from *Ricinus*, *Pterocarya*, *Onguekoa* etc. It can also be used to test the purity of acid samples and to determine the success of semihydrogenation of acetylenic acids (Crombie, 1955; Fig. 1). The amounts of material required are small (less than 5 mg. is sufficient for a complete analysis involving both oxidation and hydrogenation) and the method is therefore particularly useful with material containing only a low percentage of fat, or with material difficult to obtain in quantity, such as fungal spores. The method has obvious possibilities for radioautographic work with ^{14}C-labelled fatty acids.

SUMMARY

1. The separation of over 40 pure fatty acids on several reversed-phase paper-chromatographic systems is described, and the relationship between structure and R_F is discussed. For acids of chain length C_{12}–C_{22} (saturated and unsaturated) the best results were obtained with a paraffin/aqueous acetic acid system. For acids containing more than one hydroxyl group a castor oil/aqueous acetic acid system gives satisfactory results.

2. The method is made quantitative by photometric estimation of the copper (as the dithiooxamide complex) retained in the paper by the copper soaps of the fatty acids. Amounts of acid from 1 to 70 μg. in each spot and total quantities of up to 140 μg. of mixed fatty acids can be estimated.

3. A method is described for the analysis of natural mixtures of saturated and unsaturated acids by comparison of chromatograms of: (1) the original mixture, (2) the mixture after hydrogenation, and (3) the mixture after oxidation with alkaline permanganate. For a full analysis 5 mg. of fatty acids is sufficient.

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Effects of Methyl Bromide on Phosphorus Metabolism in the Adult Housefly, *Musca domestica* L.

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The distribution and biochemical significance of the acid-soluble phosphorus compounds of insect tissues closely resemble those of higher animals. For example, the principal intermediates which accumulate in rat muscle (LePage, 1948) are also those found in the thoracic muscle of the housefly (Winteringham, Bridges & Hellyer, 1955*a*). Chefurka (1954) has demonstrated in homogenates of housefly tissue many of the glycolytic enzymes of the Embden–Meyerhof scheme. Oxidative phosphorylation has been demonstrated with sarcosomes isolated from blowfly muscle by Lewis & Slater (1954). Adenosine triphosphate (ATP) recovered from various insect species appears to be identical with that recovered from vertebrates (Albaum & Kletzkin, 1948; Calaby, 1951). It also appears to play a comparable role in muscle contraction (Gilmour & Calaby, 1953*a*; Maruyama, 1954*a*, *b*) and in the synthesis of acetylcholine (Smallman, 1956). There are, however, some differences between the phosphorus metabolism of insects and higher animals which might be significant in the mechanisms of toxic action in the intact animal. For example, Sacktor (1955) and Zebe & McShan (1957) have observed a remarkably low lactic acid dehydrogenase activity in some insect-muscle preparations and have suggested that lactic acid may not accumulate as an end product of glycolysis as it does in vertebrate skeletal muscle. An earlier intermediate such as a triose phosphate may enter the Krebs cycle more directly. Arginine phosphoric acid (Arg-P) plays the part of the phosphagen of insects, in common with other invertebrates (Baldwin & Needham, 1934), instead of the creatine phosphoric acid of vertebrates. There is evidence that certain of the glycolytic enzymes of insect and vertebrate tissues may differ in their sensitivity to inhibitors. For example, Chefurka (1954) was unable to find any inhibition of housefly triose phosphate dehydrogenase by 0·1 mM-iodoacetate, which inhibits the mammalian enzyme (Cori, Slein & Cori, 1948). This paper describes a quantitative study, by means of the labelled-pool technique (Winteringham, 1956), of the effects of methyl bromide on the phosphorus metabolism of the adult housefly, *Musca domestica* L., *in vivo*. For a preliminary communication see Winteringham & Hellyer (1954).

METHODS AND MATERIALS

Labelling and extraction. Techniques (Winteringham *et al.* 1955*a*) for labelling with ^{32}P the soluble intermediates of adult houseflies *in vivo*, and for their extraction, have been used with minor modifications. Entire thoraces (minus wing and leg appendages) or abdomens have been homogenized and extracted three times with 1·05 ml. portions of ethanolic formic acid instead of 1·8 ml. portions as originally described, and concentration of the extracts *in vacuo* has been omitted. This modification was without significant effect on either recovery or distribution of the labelled compounds. Entire heads have similarly been extracted three times with 0·55 ml. portions of ethanolic formic acid.

Paper-radiochromatographic techniques. The techniques described earlier (Winteringham *et al.* 1955*a*) have been used with slight modification. Resolution of the ATP-adenosine diphosphate (ADP) fraction in the aq. acetic acid–acetone solvent originally described has been omitted in the

more recent work. It was found that when ADP occurred in significant proportions it was sufficiently well separated in the initial run in the aq. formic acid–acetone solvent, with a mean R_F value of 0·24 compared with the mean value of 0·13 for ATP. In cases of doubt unlabelled ADP was added as carrier before chromatography and located either chemically or in ultraviolet light on the final paper chromatograms. By a determination of the proportion of the total ^{32}P activity within the located boundaries of the added carrier it was possible to set an upper limit for the corresponding labelled compound recovered from the insect. This has been indicated in the results below by $>$. A similar procedure has been adopted for other compounds in cases of incomplete resolution. In some experiments no attempt was made to resolve a composite fraction and the principal constituents of such fractions have been bracketed together in the tabulated results (see below).

Chemical identity of labelled fractions. Further hydrolysis and cochromatographic experiments with authentic compounds have confirmed the identities of the principal fractions reported earlier (Winteringham *et al.* 1955*a*), but the description of a fraction as a particular compound must not be taken to exclude the presence of other unidentified compounds in relatively small but possibly variable proportions. For example, in the ATP fraction there was an unidentified compound (UC. 1) which on hydrolysis for 10 min. in N-HCl liberated less of its ^{32}P activity as inorganic phosphate (PO_4^{3-}) than did ATP under the same conditions. In the ammoniacal methanol solvent used for resolving the composite fraction containing arginine phosphoric acid (Arg-P), adenylic acid (AMP) and glucose 6-phosphate (G 6-P), a second fraction (UC. 2) ran with AMP. This was not inosinic acid (IMP). A third compound (UC. 3) occurring chiefly in abdominal extracts, ran faster than G 6-P in ammoniacal methanol but was not fructose 6-phosphate. A fourth fraction (UC. 4) ran faster than the PO_4^{3-} fraction in the aq. formic acid–acetone solvent or was absent. In a few cases there was evidence that UC. 4 was present in a relatively small proportion and incompletely separated from the PO_4^{3-} fraction. This has been indicated by brackets in Table 3. The recent application of these techniques to the study of the distribution of phosphorus in sarcosomes isolated from the adult blowfly, *Calliphora erythrocephala*, suggests that UC. 4 is a phospholipid associated with the sarcosomes of the thoracic tissues and liberates glycerophosphate on hydrolysis. UC. 1 may similarly be coenzyme I, which also appears to be associated with the sarcosomes and represents less than 2% of the total soluble phosphorus of the blowfly thorax (S. E. Lewis & G. M. Price, personal communication).

Insects and their treatment. Female adult houseflies (*Musca domestica* L.), 3–8 days old at the time of extraction, were used throughout. The flies were drawn from the Laboratory stock, which has been maintained by continuous breeding over a period of years. All experiments were conducted at 30°. For studying the effects of toxic vapours groups of 10 flies, which had been fed with $^{32}PO_4^{3-}$, were anaesthetized for 2 min. with *cyclo*propane, transferred rapidly to a closed stainless-steel-gauze cage, allowed to recover completely and then immersed for the required time in a jar containing air saturated with the vapour. The exposed flies were then observed in fresh air or were immediately plunged into liquid nitrogen for dissection and extraction.

RESULTS

Mean distributions of phosphorus in normal housefly tissues. These have been determined in 49 groups of 10 adult female houseflies representing 30 different generations. The distribution of phosphorus in any extract has been taken as the mean of at least two paper-radio-chromatographic analyses (see below). The mean distributions found in the thoracic extracts (representing flight muscle), with the ranges and standard deviations, are summarized in Table 1. The mean distributions found in the head and abdominal extracts are based on 11 and 7 groups respectively. It is assumed on the basis of uniformity of labelling (see Winteringham *et al.* 1955*a*) that the distribution of ^{32}P will be the same as that of the natural phosphorus. In all cases the insects were 'labelled' and 'metabolized' for 24 hr. periods at 30° and, where necessary, brought to a state of rest by *cyclo*propane anaesthesia for 2 min. The mean distribution of total soluble phosphorus between the different body regions is also shown in Table 1.

Variation in replicate analyses. In order to test the reproducibility of the technique for the analysis of the labelled fractions 20 radio-chromatographic analyses was made on the same extract under routine conditions. In practice at least two analyses are made on each extract, so that the calculated standard error of the means of all possible pairs of results for a particular fraction will indicate the order of variation to be expected as a result of the analytical technique itself. The results are summarized in Table 2. The extract used in this test had been prepared from a group of flies which had been exposed to continuous *cyclo*propane anaesthesia for some hours, and accounts for the low ATP value.

Significance of results. The distributions found in the tissues of the treated insects have been compared with those found in normal insects (Table 1). Significance has been assessed by the t test and the corresponding level of probability indicated. Two groups of insects were used in experiments to study the effects of a toxic substance: a control group and a treated group whose treatments were identical except for exposure to the toxic substance. The control group provided a check on the condition of the insects used for the experiment and on the possible effects of the additional manipulation associated with exposure to the toxic substance. The results for a control group might therefore differ significantly from those of Table 1. For example, if an indirect result of exposure to the toxic substance was the inability of the insect to feed then the control insects would also be starved so that indirect effects of starvation might be assessed. In four instances the control-group

Table 1. *Distribution of soluble phosphorus in tissues of the adult housefly*

Fraction	^{32}P of fraction as % of the total soluble $^{32}P \pm$ S.D.	Range
Head		
ATP + UC. 1	35·9 ± 6·8	25·5–45·8
ADP	4·9 ± 2·2	0 – 7·6
AMP + UC. 2	4·3 ± 1·4	1·9– 6·6
G 6-P	10·6 ± 2·8	6·7–15·2
UC. 3	0·8	0 – 3·1
Arg-P	6·2 ± 1·7	3·4– 8·5
PGA	13·0 ± 3·8	8·8–18·5
PO_4^{3-}	20·9 ± 4·2	13·4–28·7
UC. 4	3·4	0 – 9·5
Thorax		
ATP + UC. 1	34·7 ± 4·8	25·4–44·9
ADP	3·7 ± 2·4	0 –11·4
AMP + UC. 2	3·6 ± 1·9	0 –11·0
G 6-P	9·1 ± 3·2	3·6–17·9
UC. 3	Trace	—
Arg-P	10·5 ± 3·4	2·8–19·0
PGA	20·9 ± 4·6	11·2–33·1
PO_4^{3-}	16·8 ± 4·7	6·7–27·8
UC. 4	0·7	0 – 7·0
Abdomen		
ATP + UC. 1 / ADP	21·6 ± 3·1	18·1–27·4
AMP + UC. 2 + G 6-P + UC. 3 + Arg-P	17·0 ± 1·8	13·8–19·6
PGA	4·9 ± 1·4	3·0– 7·2
PO_4^{3-} + UC. 4	56·5 ± 5·0	45·8–59·9
Mean ^{32}P in body regions as % of the total soluble ^{32}P	Head 4·7 Thorax 40·4 Abdomen 54·9	

Table 2. *Variation in replicate analyses of the same extract*

Fraction	^{32}P of fraction as % of total soluble $^{32}P \pm$ S.E. of means of pairs
ATP + ADP + UC. 1	26·0 ± 1·1
AMP + Arg-P + G 6-P + UC. 2 + UC. 3	24·1 ± 1·7
PGA	24·9 ± 1·5
PO_4^{3-} + UC. 4	25·0 ± 0·9

distributions of soluble phosphorus differed significantly from those shown in Table 1 and in such a way as to complicate the interpretation of differences found in the treated groups (see below). The thoracic ATP and ADP fractions of the control group in Expt. 4, Table 3, were significantly low and high respectively ($P < 0·01$) and the thoracic G 6-P was significantly high ($P < 0·02$) in the control groups of Expt. 7, Table 3. The levels of thoracic Arg-P were unusually high in Expts. 4 and 5, Table 3.

Effects of methyl bromide

The results are shown in Table 3. Two or more fractions have been bracketed when their resolution was inadequate or not attempted. When the insects were immobilized and not allowed to recover (Expts. 1–10) there were significant and sometimes severe depletions of head and thoracic ATP fractions ($P < 0·05$). In Expt. 4 the thoracic ATP of the control group was also significantly low (see above), but methyl bromide had nevertheless caused a highly significant ($P < 0·01$) depletion of the combined ATP + ADP fraction; the corresponding fraction of the control group did not differ significantly from the normal mean of Table 1. In Expts. 2, 3, 5, 6, 7 and 10 there were significant ($P < 0·05$) increases in thoracic AMP and corresponding increases in the PO_4^{3-} fractions. In Expt. 8 there were also significant ($P < 0·01$) increases in the head AMP and PO_4^{3-} fractions.

These results strongly suggest that immobilization of the insect was associated with a breakdown of ATP according to the equation:

$$ATP \rightarrow AMP + 2PO_4^{3-}.$$

This is shown by the data of Table 4, which are derived from the treated groups of Expts. 1–10, Table 3, and the normal groups of Table 1. It will be seen that there is reasonable agreement between

the average changes in the AMP fraction and those calculated on the basis of the above equation and on the assumption that ADP changes according to the equation $ADP \rightleftharpoons AMP + PO_4^{3-}$. The possible effects of changes in UC. 1 and UC. 2 on the results of Table 4 have been neglected. In Expt. 6 of Table 3, however, the increase in the AMP + UC. 2 fraction was shown by cochromatography with the authentic compound and by acid hydrolysis to be due entirely to AMP.

Significantly high values for thoracic G 6-P were observed in the heavily poisoned insects of Expts. 6 and 7 ($P < 0.01$) and in Expt. 9 ($P < 0.05$). There was evidence therefore that excessive doses of methyl bromide may cause an increase in the level of thoracic G 6-P but this effect was not observed consistently (cf. Expt. 10, Table 3).

Except in Expts. 4 and 5 the level of thoracic Arg-P was reduced by exposure to methyl bromide when the insects were not allowed to recover (Expts. 1–10) and was apparently associated with the fall in ATP, the largest effect on Arg-P being observed in Expt. 7, which showed the largest depletion of ATP. If, on the other hand, the unusually high levels of thoracic Arg-P in the control groups of Expts. 4 and 5 are accepted the results of these two experiments also fall into line. Significant ($P < 0.05$) and highly significant ($P < 0.01$) falls in the levels of thoracic phosphoglycerate (PGA) were observed only after excessive exposures to methyl bromide of 10 min. or longer (Expts. 6, 8, 9 and 10). The reduction of the level of PGA clearly proceeded more slowly than that of the high-energy phosphates ATP and Arg-P. The free inorganic PO_4^{3-} fraction of head, thoracic or abdominal tissues was always increased as the result of the breakdown of ATP and Arg-P.

Effects of ethylene dichloride and iodoacetic acid

The results of some experiments with ethylene dichloride (1:2-dichloroethane) vapour and iodoacetic acid are shown in Table 5. It will be seen that narcosis with ethylene dichloride had little effect on the distribution of thoracic phosphorus except for an increase in the free inorganic phosphate fraction. Iodoacetate, on the other hand, caused marked and significant depletions of ATP and PGA in thoracic tissues, the depletion of PGA being consistent with iodoacetate acting as an inhibitor of triose phosphate dehydrogenase. There was a consequent accumulation of free inorganic phosphate. Significant ($P < 0.02$) accumulations of thoracic G 6-P were consistent with the early observation of Lundsgaard (1930) that a hexose phosphate accumulated in the iodoacetate-poisoned muscle of the frog. The breakdown of thoracic ATP in the iodoacetate-poisoned fly was accompanied by the formation of AMP. ATP was significantly reduced in the head tissues ($P < 0.05$). The treated head and control thoracic Arg-P were significantly high ($P < 0.02$) in Expt. 3. This may have been connected with the fact that both groups in this experiment had also received relatively massive injections of fluid, the effect being masked in the treated thorax by the locol action of the iodoacetate.

DISCUSSION

Methyl bromide readily and irreversibly methylates certain free and protein amino acids, especially the SH groups of cysteine and reduced glutathione, the $S \cdot CH_3$ group of methionine, and the imidazole group of histidine (Blackburn, Consden & Phillips, 1944; Winteringham & Harrison, 1946; Lewis & Eccleston, 1946; Lewis, 1948; Winteringham, Harrison, Bridges & Bridges, 1955*b*; Bridges, 1955).

The rapid blocking of the free SH groups of the tissues of *Calliphora erythrocephala* larvae *in vivo* by high concentrations of methyl bromide vapour has been demonstrated (Loveday & Winteringham, 1951). Consistent with these findings is the fact that methyl bromide has been shown to inhibit several SH enzymes *in vitro*, including yeast hexokinase (Dixon & Needham, 1946), urease and mammalian succinic dehydrogenase (Lewis, 1948). The wide dependence of glycolysis and pyruvate oxidation on SH enzymes and the SH groups of cofactors like coenzyme A and the reduced form of lipoic acid suggest that methyl bromide might be expected profoundly to disturb basal metabolism *in vivo* and to resemble in its action other SH reagents such as iodoacetate. It remains to discuss the present results against this background.

Typically, when adult houseflies are exposed to a lethal but not excessive dose of methyl bromide vapour in air, as in Expts. 1–3 and 11–13 of Table 3, they immediately collapse and appear to be deeply anaesthetized. On removal to fresh air they recover within minutes and behave normally for 1–3 hr. when, invariably, they collapse again and fail to recover. After the initial exposure to methyl bromide the flies respire at a normal or slightly supranormal rate for several hours. Respiration then gradually falls off. When the flies are exposed to an excessive lethal dose (as in Expts. 6–10, Table 3) there is no temporary recovery in fresh air and respiration falls off immediately. This is illustrated by the results shown in Fig. 1 (F. P. W. Winteringham & A. Weatherley, unpublished work). Similar observations have been made by Bond (1956), who found that the oxygen uptake of larvae or adults of *Tenebroides mauritanicus* was unaffected during exposure to LD_{99} doses of methyl bromide vapour, despite a considerable

Table 3. *Effects of methyl bromide on the distribution of the soluble phosphorus in the tissues of the adult housefly* in vivo

Expt. no.	Treatment and signs of poisoning at the time of freezing and extraction	Fraction	^{32}P of fraction as % of total soluble ^{32}P recovered: Head Control	Head Test	Thorax Control	Thorax Test	Abdomen Control	Abdomen Test
1*	1 min. exposure to methyl bromide; complete collapse; immediate extraction. Similar unextracted group recovered within minutes but majority collapsed again some hours later and never recovered	ATP + UC. 1			36·3	23·0		
		ADP			0	0		
		AMP + UC. 2			25·6	6·6		
		G 6-P				14·9		
		UC. 3				0		
		Arg-P				5·7		
		PGA			21·1	19·3		
		PO_4^{3-}			17·0	30·5		
		UC. 4						
2	As in Expt. 1	ATP + UC. 1			40·0	20·8		
		ADP			0	0		
		AMP + UC. 2			5·0	9·7		
		G 6-P				10·0		
		UC. 3			0	0		
		Arg-P			18·5	7·4		
		PGA			17·6	18·7		
		PO_4^{3-}			18·9	33·4		
		UC. 4						
3*	As in Expt. 1	ATP + UC. 1			27·0	11·4		
		ADP			+ 3·2	3·1		
		AMP + UC. 2			4·1	7·5		
		G 6-P			9·7	8·4		
		UC. 3			0	0		
		Arg-P			9·2	2·8		
		PGA			16·0	15·8		
		PO_4^{3-}			25·0	49·7		
		UC. 4			5·8	1·3		
4	2 min. exposure to methyl bromide; complete collapse; immediate extraction. Similar unextracted group recovered within minutes but collapsed again some hours later and never recovered	ATP + UC. 1	33·9	19·4	21·1	18·0	14·4	10·4
		ADP	+ 4·3	8·0	10·7	+ 4·4	+ 3·7	+ 2·1
		AMP + UC. 2	3·0	4·7	2·8	3·7	1·6	1·8
		G 6-P	6·2	13·0	7·3	9·2	7·1	4·2
		UC. 3	4·0	2·8	4·6 (?)	0	7·4	4·6
		Arg-P	5·7	8·9	14·6	14·1	2·5	3·7
		PGA	10·6	7·7	19·9	11·9	3·7	3·6
		PO_4^{3-}	27·6	28·6	16·2	32·0	56·8	67·8
		UC. 4	4·7	6·9	2·8	6·7	2·8	1·8
5	5 min. exposure to methyl bromide; complete collapse; immediate extraction. Similar unextracted group failed to recover in fresh air	ATP + UC. 1			31·3	10·0		
		ADP			0	0		
		AMP + UC. 2			8·9	15·2		
		G 6-P			7·8	8·2		
		UC. 3			0	0		
		Arg-P			20·8	10·5		
		PGA			18·8	17·5		
		PO_4^{3-}			12·4	38·6		
		UC. 4			Trace	Trace		
6	10 min. exposure to methyl bromide; complete collapse; immediate extraction. Similar unextracted group failed to recover in fresh air	ATP + UC. 1			39·0	13·2		
		ADP			0	2·1		
		AMP + UC. 2			4·3	21·2		
		G 6-P			8·8	17·9		
		UC. 3			0	0		
		Arg-P			10·1	3·1		
		PGA			16·0	8·5		
		PO_4^{3-}			21·8	34·0		
		UC. 4			0	0		
7	As in Expt. 6	ATP + UC. 1			38·6	4·9		
		ADP			0	3·4		
		AMP + UC. 2			1·1	10·7		
		G 6-P			17·9	24·2		
		UC. 3			Trace	0		
		Arg-P			10·4	0		
		PGA			18·7	19·9		
		PO_4^{3-}			13·3	36·9		
		UC. 4			0	0		

Table 3 (*cont.*)

Expt. no.	Treatment and signs of poisoning at the time of freezing and extraction	Fraction	^{32}P of fraction as % of total soluble ^{32}P recovered: Head Control	Head Test	Thorax Control	Thorax Test	Abdomen Control	Abdomen Test
8	As in Expt. 6	ATP + UC. 1	45·2	8·3	31·0	7·7		
		ADP	0	5·0	6·4	2·4		
		AMP + UC. 2	3·2	12·6	3·2	5·4		
		G 6-P	10·9	6·4	6·7	5·3		
		UC. 3	Trace	Trace	0	0		
		Arg-P	3·4	3·2	10·9	3·2		
		PGA	13·2	14·2	23·6	10·7		
		PO_4^{3-} + UC. 4	24·1	50·3	18·2	65·3		
9	60 min. exposure to methyl bromide; immediate extraction. Similar unextracted group failed to recover	ATP + UC. 1			36·3	14·0		
		ADP			0	0		
		AMP + UC. 2			25·6	6·5		
		G 6-P				16·2		
		UC. 3				0		
		Arg-P				4·3		
		PGA			21·1	5·4		
		PO_4^{3-} + UC. 4			17·0	53·6		
10	120 min. exposure to methyl bromide; immediate extraction. Similar unextracted group failed to recover	ATP + UC. 1			27·0	6·6		
		ADP			‡3·2	0		
		AMP + UC. 2			4·1	11·3		
		G 6-P			9·7	6·5		
		UC. 3			0	0		
		Arg-P			9·2	7·2		
		PGA			16·0	7·0		
		PO_4^{3-}			25·0	58·8		
		UC. 4			5·8	2·6		
11*	1 min. exposure to methyl bromide; followed by 60 min. aeration before extraction. All flies had recovered from initial collapse. Similar unextracted group finally collapsed some hours later	ATP + UC. 1			40·0	35·2		
		ADP			0	0		
		AMP + UC. 2			5·0	6·8		
		G 6-P				6·5		
		UC. 3			0	0		
		Arg-P			18·5	11·9		
		PGA			17·6	8·5		
		PO_4^{3-} + UC. 4			18·9	31·1		
12	2 min. exposure to methyl bromide; followed by aeration. All flies recovered but collapsed 2 hr. or more later. Each fly was frozen and extracted on final collapse and an extract of 10 thoraces was pooled	ATP + UC. 1			27·0	29·9		
		ADP			‡3·2	‡2·7		
		AMP + UC. 2			4·1	4·7		
		G 6-P			9·7	4·9		
		UC. 3			0	0		
		Arg-P			9·2	12·2		
		PGA			16·0	13·9		
		PO_4^{3-}			25·0	26·8		
		UC. 4			5·8	4·9		
13*	1 min. exposure to methyl bromide; followed by aeration. All flies recovered but collapsed 1 hr. or more later. Each fly was frozen and extracted 3 hr. after final collapse	ATP + UC. 1	32·8	18·7	31·3	17·8	24·2	15·1
		ADP	‡3·8	‡5·9	‡3·5	‡3·8	‡3·2	‡3·9
		AMP + UC. 2	1·9	3·8	3·7	4·7	0·8	1·9
		G 6-P	13·3	8·2	7·8	10·4	7·3	5·3
		UC. 3	0	3·4	0	0	5·2	4·3
		Arg-P	8·1	10·6	16·3	9·5	6·3	6·1
		PGA	18·5	11·3	19·6	9·7	7·2	6·6
		PO_4^{3-}	20·0	38·1	17·8	43·4	45·5	56·8
		UC. 4	1·6	0	0	0·7	0·3	0

* A 1 min. exposure to methyl bromide was not invariably lethal. One or two insects of an exposed group failed to undergo the delayed collapse and survived. These insects were rejected.

reduction in muscular activity, and that respiration fell off only gradually thereafter.

It will be seen from Table 3 that immediately after exposing adult houseflies to methyl bromide for 1 min. there was a fall in the thoracic ATP level. This would account for the observed prostration of the insect at this stage, and differs from anaesthetic action because houseflies may be deeply anaesthetized with *cyclo*propane without significantly affecting the level of thoracic ATP (Winteringham *et al.* 1955*a*). The ATP level of flies completely immobilized by exposure to the vapour of ethylene dichloride for 6 min. was also unaffected, as shown in Table 5. Another difference between anaesthetized flies and those immobilized with methyl bromide was that the legs and wings of the former would still respond to a single direct electrical

Table 4. *Effect of methyl bromide on tissue nucleotides of the adult housefly* in vivo

Changes are expressed as percentage of total soluble ^{32}P.

	Thorax	Head
Average observed change in ATP	−21·7	−22·0
ADP	− 2·2	+ 1·6
AMP	+ 6·2	+ 4·4
Calculated change in AMP	+ 8·3	+ 6·5

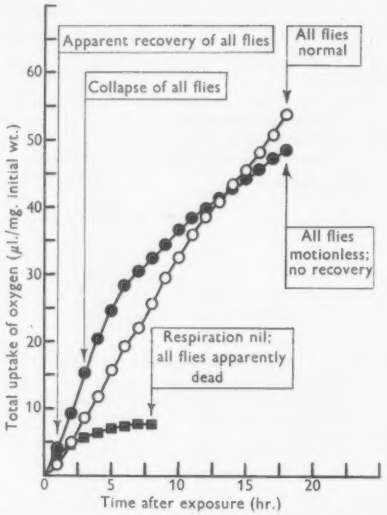

Fig. 1. Effects of just-lethal and excessive doses of methyl bromide vapour on the oxygen uptake of the adult housefly. ○, Control; ●, 1 min. exposure; ■, 15 min. exposure.

Table 5. *Effects of ethylene dichloride vapour and injected iodoacetic acid on the distribution of the soluble phosphorus in the tissues of the adult housefly* in vivo

^{32}P of fraction as % of total soluble ^{32}P recovered from body region

Expt. no.	Treatment and signs of poisoning at the time of freezing and extraction	Fraction	Head		Thorax		Abdomen	
			Control	Test	Control	Test	Control	Test
1	6 min. exposure in air saturated with ethylene dichloride vapour. All flies were motionless after 5 min.; immediate extraction. Similar unextracted group recovered completely in fresh air and showed no delayed effects	ATP + UC. 1			38·4	35·1		
		ADP			0	0		
		AMP + UC. 2			4·7	4·0		
		G 6-P			17·1	9·0		
		UC. 3			0	0		
		Arg-P			9·1	5·6		
		PGA			17·9	15·9		
		PO_4^{3-}			12·8	30·4		
		UC. 4						
2	Injected intrathoracically with 5 μg. of iodoacetic acid in 1 μl. of acetone. Flies prostrated after about 2 hr. were immediately extracted. Remaining flies survived and were rejected	ATP + UC. 1			36·7	17·0		
		ADP			0	0		
		AMP + UC. 2			0	6·5		
		G 6-P			11·6	16·6		
		UC. 3			0	0		
		Arg-P			13·5	6·3		
		PGA			15·1	5·8		
		PO_4^{3-}			23·1	47·8		
		UC. 4						
3	Injected intrathoracically with 10 μg. of iodoacetic acid in 2 μl. of water. All flies were motionless after 1 hr.; extracted 2 hr. after injection	ATP + UC. 1	31·9	20·8	30·5	11·8	22·5	25·1
		ADP		✳ 7·3	✳ 3·7	0	0	2·2
		AMP + UC. 2		3·7	1·8	10·0		
		G 6-P	22·9	7·4	6·5	18·9	13·8	21·0
		UC. 3		—	0	0		
		Arg-P		21·6	19·0	8·4		
		PGA	17·6	9·1	15·1	6·8	4·6	7·2
		PO_4^{3-}	19·4	26·7	20·4	42·6	59·1	44·5
		UC. 4	8·2	3·4	0	1·5	0	0

stimulus, whereas those of the latter would not, presumably because of the depletion of muscle ATP (Winteringham & Bridges, 1955). A possible explanation of the initial fall in ATP was acute anoxia in the presence of a high concentration of methyl bromide vapour. Anoxia caused a severe depletion of ATP and phosphagen in rabbits (Marcotte-Boy & Cheymol, 1953). On the other hand, the level of thoracic ATP was little affected in flies which were drowned at room temperature by submerging them in water containing a wetting agent until all struggles and movement ceased. The fall in ATP as a result of exposure to methyl bromide thus suggests an inhibition of phosphorylation. The corresponding formation of thoracic AMP and PO_4^{3-} but not ADP (see Table 4) would be explained by the combined action of adenosine triphosphatase and myokinase (Sacktor, 1953; Gilmour & Calaby, 1953*b*; Maruyama, 1954*b*). The absence of chromatographic evidence of formation of IMP may be due to the absence of an insect adenylic acid deaminase (Gilmour & Calaby, 1953*a*). The parallel recovery of ATP with the activity of the insect (Expt. 11, Table 3) on removal to fresh air is not so readily explained in view of the irreversible nature of alkylation and enzyme inhibition by methyl bromide.

Considering the delayed collapse following the 1 or 2 min. exposures, it will be seen that when the flies finally collapsed (Expt. 12, Table 3) the distribution of thoracic phosphorus was not markedly affected except for an increase in PO_4^{3-} of borderline significance ($P=0{\cdot}05$). Respiration was also normal at this stage (Fig. 1). The delayed collapse cannot then be explained on the basis of the inhibition *in vivo* of SH enzymes of glycolysis and respiration or the blocking of coenzyme A or lipoic acid function. This is also consistent with the fact that the proportion of $^{14}CO_2$ recovered in 24 hr. (45 %) from intact flies which had been intrathoracically injected with [2-^{14}C]acetate at the time of the final collapse was actually higher than that (38 %) recovered from flies which had not been exposed to methyl bromide (F. P. W. Winteringham, unpublished work). The possibility of there being a local inhibition of glycolysis at the triose phosphate level in nerve tissue is not supported by the fact that in Expt. 13, Table 3 there was no significant effect on the PGA of the head, which is relatively rich in nerve tissue and poor in muscle.

The action of the brief (1–2 min.) but lethal exposures of the insects to methyl bromide does not resemble that of iodoacetate, which inhibits glycolysis at the triose phosphate dehydrogenase level (Cori *et al.* 1948). When adult houseflies were injected intrathoracically with a lethal but not excessive dose of iodoacetic acid in acetone or water (Table 5) the majority of flies collapsed within about 2 hr. and failed to recover. Respiration similarly fell off rapidly.

Iodoacetate caused significant ($P<0{\cdot}01$) reductions in thoracic ATP and PGA in immobilized insects, the latter reduction being an expected consequence of triose phosphate dehydrogenase inhibition (Expts. 2 and 3, Table 5). This is in agreement with Bettini & Boccacci (1954, 1955, 1956*a*, *b*). They were able closely to relate insect mortality due to iodoacetate with the inhibition of triose phosphate dehydrogenase *in vivo* and *in vitro* but not with the inhibition of succinic dehydrogenase or with the blocking of coenzyme A function, and this despite the apparent insensitivity of insect triose phosphate dehydrogenase to iodoacetate reported by Chefurka (1954). An effective reduction in insects of carbohydrate and pyruvate oxidation by iodoacetate would be expected from the classical work with vertebrate tissues by Lundsgaard (1931) and by Peters and his colleagues (see Peters & Wakelin, 1946). The expected reduction in oxidative phosphorylation combined with the insensitivity of insect actinomyosin adenosine triphosphatase and soluble adenosine triphosphatase to iodoacetate (Maruyama, 1954*b*) would then account for the fall in ATP shown in Table 5.

Excessive doses of methyl bromide, from which the insects never recovered (Expts. 6–10, Table 3), usually caused a reduction in thoracic ATP, Arg-P and PGA with corresponding increases in PO_4^{3-}, but the pattern of changes in the head tissues of the same insects was not necessarily similar (Expt. 8, Table 3). Respiration also ceased (Fig. 1). The action of excessive doses of methyl bromide may therefore be to block SH enzymes and contingent respiration and so resemble the action of iodoacetate.

The results of Expt. 13 (Table 3) show that 3 hr. after the final collapse after just-lethal exposure to methyl bromide there were delayed but significant depletions of ATP ($P<0{\cdot}01$) and of PGA ($P<0{\cdot}05$), but these appeared to be too delayed to account for the final collapse of the insect (cf. Expt. 12, Table 3). Such delayed falls in ATP, PGA and other phosphorylated intermediates have been observed, moreover, in flies exposed to lethal doses of non-SH-reactive substances, and to extreme starvation, and may be non-specific and indirect consequences of the dying cell.

This study provides no evidence that the immediate and delayed signs of poisoning in the adult housefly briefly exposed to a lethal but not excessive dose of methyl bromide vapour can be explained in terms of SH enzyme inhibition or the blocking of the SH groups of coenzyme A or reduced lipoic acid. The action of lethal but not excessive doses of methyl bromide in the adult

housefly apparently differs therefore from that of iodoacetate. There is evidence, however, that excessive doses of methyl bromide resemble iodoacetate in their action as a triose phosphate dehydrogenase inhibitor and cause a marked and irreversible depletion of phosphoglycerate and muscle ATP *in vivo*.

SUMMARY

1. Adult female houseflies were exposed to a saturation concentration of methyl bromide vapour for 1 min. (lethal but not excessive exposures) or for longer periods (excessive exposures) at 30°.

2. Insects exposed for 1 or 2 min. were immobilized but recovered temporarily in fresh air. These insects finally collapsed again 1–3 hr. later and failed to recover. Respiration continued at a near normal rate for several hours after the delayed collapse.

3. Initial immobilization of the insect by brief exposure to methyl bromide was apparently due to a reversible breakdown of adenosine triphosphate (ATP) according to the overall equation: ATP $\rightleftharpoons$ adenosine 5′-phosphate + $2PO_4^{3-}$.

4. Excessive exposures to methyl bromide immediately and irreversibly immobilized the insect. Such exposures were associated with depletions of ATP, arginine phosphoric acid and phosphoglyceric acid.

5. Temporary recovery of the insects after brief exposures was associated with a return towards normal levels of thoracic ATP.

6. The reversible effects of brief exposures on the level of ATP, the delayed collapse of the insect without marked changes in distribution of soluble phosphorus and the sustained respiration were not consistent with the action of methyl bromide as an SH-enzyme inhibitor.

7. The effects of excessive exposures to methyl bromide resembled those of lethal but not excessive doses of iodoacetate characterized by depletion of ATP and phosphoglycerate in the thoracic tissues.

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Acidic Peptides of the Lens

4. THE BIOSYNTHESIS OF OPHTHALMIC ACID*

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Ophthalmic acid, a tripeptide isolated from calf lens (Waley, 1956, 1957), has been shown to have the structure (I; R=CH_3) (Waley, 1958). This structure is very similar to that of glutathione (I; R=SH), and so the question naturally arises whether the two peptides are formed *in vivo* by the same route.

$$H_2N\cdot CH(CO_2H)\cdot[CH_2]_2\cdot CO\cdot NH\cdot CH(CH_2R)\cdot CO\cdot NH\cdot CH_2\cdot CO_2H$$

(I)

The synthesis of glutathione in liver extracts takes place in two stages, each catalysed by a separate enzyme (Snoke & Bloch, 1952; Snoke, Yanari & Bloch, 1953; Yanari, Snoke & Bloch, 1953):

Glutamic acid + cysteine + ATP → γ-glutamyl cysteine + ADP + H_3PO_4

γ-Glutamyl cysteine + glycine + ATP → glutathione + ADP + H_3PO_4

(ATP, adenosine triphosphate; ADP, adenosine diphosphate).

Lens extracts catalyse the synthesis of glutathione from the three amino acids and ATP (Daisley, 1955), presumably by the same two stages. This communication describes the synthesis of ophthalmic acid from its three amino acids and ATP in lens extracts; this synthesis also proceeds by a two-stage mechanism; moreover, at least one of the enzymes of the synthesis of glutathione also plays a part in the synthesis of ophthalmic acid.

EXPERIMENTAL

Materials

Lens preparations. Lenses were extracted from the eyes of calves within 2 hr. of the death of the animals; they could be stored in a frozen state for at least 4 weeks without impairment of enzymic activity. The lenses of rabbits (Dutch, 3–4 months old) were used fresh. The average weight of a calf lens is 1 g., and of a rabbit lens 0·35 g. Two types of lens preparation were used. In the first type (called whole lens), the lenses were ground at 4° with an equal weight of water and a little sand; the mixture was not centrifuged. In the second type of preparation (called dialysed lens), this whole lens preparation was dialysed at 4° for 24 hr. against water, and then for 24 hr. against 0·005 M-potassium phosphate, pH 6·4. The membrane was cellulose dialysis tubing, 0·75 in. in diameter (Gallenkamp and Co., London); the diffusate sacks were inverted, and the outer liquid renewed, every 2 hr. during the day. Thorough dialysis was necessary to remove most of the glutathione from the lens mixture. The contents of the dialysis sack were centrifuged for 20 min. at 4000 *g* at 4°, and the supernatant (1·7 ml. from 1 g. of lens) was used.

Peptides. Ophthalmic acid, norophthalmic acid, γ-L-glutamyl-L-α-amino-*n*-butyric acid and L-α-amino-*n*-butyrylglycine were prepared as previously described (Waley, 1958), and the glutathione was from The Distillers Co. Ltd.

Co-factors. The barium salt of D-3-phosphoglyceric acid (Light and Co., Colnbrook, Bucks.) was converted into the potassium salt with Amberlite IR-120(H^+) and KOH (Wade & Morgan, 1954).

Methods

The reactions were followed by paper electrophoresis. There are many advantages in following reactions catalysed by crude tissue extracts by an analytical method in which the products are separated and identified, and this is particularly important when several reactions are proceeding concurrently (cf. Waley & Watson, 1953, 1954). Paper electrophoresis was preferred to paper chromatography mainly because compact, intense spots with a low background colour were obtained by the procedure described below.

System used for synthesis of peptides. The concentrations of amino acids (or peptides) were 8 mM, except that the concentration of DL-α-amino-*n*-butyric acid was 16 mM. The mixture also contained ATP (Sigma Chemical Co.) (2·5 mM), 3-phosphoglyceric acid (22 mM), KCl (42 mM), $MgSO_4$ (10 mM) and potassium phosphate buffer, pH 6·4 (6 mM). The total volume of the solution was 10 ml., and it contained 7·7 ml. of calf-lens preparation (the experiments with rabbit lens were done on a smaller scale); the reaction was carried out (at pH 6·3) at 37° for 5 hr.; samples were withdrawn at intervals; after removal of protein and evaporation, the residues, in 10% (v/v) propan-2-ol, were examined by paper electrophoresis.

Removal of protein. Samples were taken at intervals from the reaction mixture and protein was precipitated by the addition of 2·5 vol. of hot ethanol; a measured fraction of the supernatant was evaporated *in vacuo* and the residue dissolved in 10% (v/v) propan-2-ol. The protein precipitate is bulky and estimations with the ninhydrin reagent of Troll & Cannan (1953) (after known amounts of ophthalmic acid had been added) showed that the amount of ophthalmic acid in the supernatant was only 87% of that expected, owing to adsorption on the protein precipitate. Hence this correction factor has been applied throughout.

* Part 3: Waley (1958).

Removal of glutathione. The electrophoretic mobility of reduced glutathione (GSH) is the same as that of ophthalmic acid, hence the necessity for the thorough dialysis described above. In some experiments in which GSH was necessarily present in the incubation mixture, the GSH was oxidized either to the disulphide (GSSG) or to the sulphonic acid (GSO_3H). Autoxidation of the GSH to GSSG was effected at pH 8 by dissolving the residue (after removal of proteins) in 0·2 M-$KHCO_3$ and allowing the solution to stand in open tubes for 3 hr., with occasional agitation to ensure adequate access of oxygen. The electrophoretic mobility of GSSG is greater than that of GSH, as would be expected from the titration curves (Pirie & Pinhey, 1929). Oxidation of GSH to GSO_3H with performic acid was carried out as described previously (Waley, 1956); ophthalmic acid was found to be unaffected by performic acid.

Paper electrophoresis. A ridge-pole apparatus was used (Durrum, 1950); the apex height was 11·5 cm., and the paper sheets (Whatman no. 52) were 25 cm. long (measuring from one electrode to the other), and up to 44 cm. wide. The paper was soaked in a pyridine acetate buffer (50 ml. of acetic acid, 15 ml. of pyridine and 2·5 l. of water; Grassmann, Hannig & Plöckl, 1955), blotted and laid between polythene sheets so as to leave exposed only the area where the spots were to be put on. This prevented the rest of the paper from drying while the samples (5 μl.) were applied to the starting line 5 cm. from the cathode end. Electrophoresis was carried out at 8 v/cm. for 8 hr. to separate glutamic acid and ophthalmic acid, and for 3·5 hr. when it was necessary to prevent 5-oxopyrrolidine-2-carboxylic acid from migrating off the paper. The dried paper was dipped (six times) through ninhydrin (0·4%, w/v) and cobalt chloride (0·2%, w/v) in propan-2-ol (Wiggins & Williams, 1952) and excess of reagent allowed to drain off; the paper was heated at 90° for 5 min., held in a jet of steam for 3 min. (exposing the whole surface on both sides to the steam), and then left in the dark for 48 hr. The intensity of the spots was measured as previously described (Waley, 1957). Standard solutions have to be run on each sheet; a calibration curve is shown in Fig. 1; 5 μl. of a mM-solution (5 μm-moles) can be estimated with an error of about 5%.

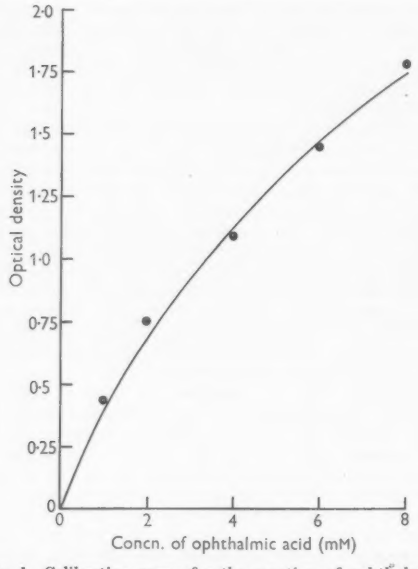

Fig. 1. Calibration curve for the reaction of ophthalmic acid with ninhydrin, measured on paper.

RESULTS

Units of rate

The unit of substrate or product concentration is μM, of enzyme concentration grams (wet weight) of tissue/ml., and the unit of time is the minute; all rates given are expressed in these units, which for brevity are omitted below.

Synthesis of ophthalmic acid from its constituent amino acids

The system was similar to that used by Johnson & Bloch (1951) for the synthesis of glutathione; they found it necessary to reconvert the ADP formed during peptide-bond synthesis into ATP, as ADP inhibited the synthesis of glutathione, and so they added 3-phosphoglyceric acid and a muscle preparation. Even dilute lens extracts catalyse the phosphorylation of ADP by 3-phosphoglyceric acid, so that the muscle preparation can be omitted in the synthesis of glutathione or ophthalmic acid by lens extracts.

The product of the reaction was identified as ophthalmic acid by its chromatographic and electrophoretic mobility. This was confirmed by isolation. The product was eluted from the electrophoresis paper and further purified by preparative paper chromatography in 72% (w/w) phenol–3% (w/v) NH_3 soln. (Waley, 1957). Hydrolysis, followed by quantitative paper chromatography, gave the molar ratios of the amino acids in the hydrolysate as glycine 1·0, α-amino-*n*-butyric acid 1·1, glutamic acid 1·0. Moreover, comparison of the X-ray powder photograph of the copper salt with that of an authentic specimen (for which we are indebted to Mrs D. M. Hodgkin, F.R.S., and Dr B. M. Oughton) confirmed that the product was ophthalmic acid.

The rates of synthesis of ophthalmic acid in several experiments are given in Table 1; the rates did not change appreciably during the reactions, at least during the early stages. The rate varied somewhat with different batches of lenses, and so the same batch was used for any important comparison, as is shown in Table 1. The rate of synthesis of ophthalmic acid was the same whether DL-α-amino-*n*-butyric acid (16 mM) or L-α-amino-*n*-butyric acid (8 mM) was used; hence probably only

the L-form is utilized, and the D-form does not compete under these conditions. The rate of synthesis was about the same whether 'whole lens' or 'dialysed lens' preparations from either the calf or the rabbit were used.

Interaction between the synthesis of ophthalmic acid and of glutathione. Lens batches A and B were used to investigate competition when both ophthalmic acid and glutathione are being synthesized simultaneously. When the concentration of L-cysteine (freshly prepared by neutralization of the hydrochloride) was 8 mM the rate of synthesis of ophthalmic acid was appreciably reduced (Fig. 2; Table 1); in this experiment the GSH synthesized was oxidized as described to GSSG before electrophoresis.

Table 1. *Rates of synthesis of ophthalmic acid*

Each tube contained L-glutamic acid (8 mM), glycine (8 mM) and DL-α-amino-*n*-butyric acid (16 mM), and dialysed lens preparation, except for the last two experiments given in the fourth column, where whole-lens preparations were used. For units of rate see text. The probable errors given are obtained from fitting the best straight lines to the experimental points.

Lens batch	Animal	Rate: Standard conditions	Rate: Cysteine added
A	Calf	8·7 (±0·8)	4·7 (±0·4)
B	Calf	10·3 (±1·2)	3·1 (±0·5)
		Whole lens	
C	Calf	14·3	10·9
D	Rabbit	13·8	13·8

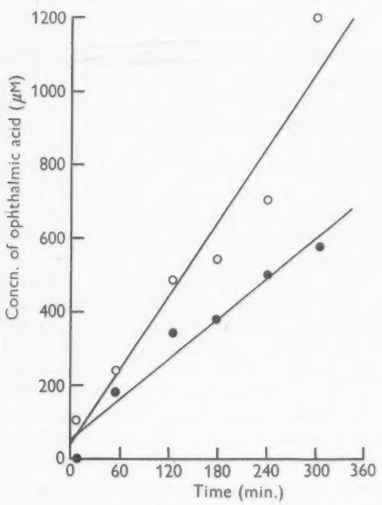

Fig. 2. Formation of ophthalmic acid in the presence (●) and absence (○) of cysteine, followed by paper electrophoresis.

Formation of 5-oxopyrrolidine-2-carboxylic acid. 5-Oxopyrrolidine-2-carboxylic acid is also formed in the course of the synthesis of ophthalmic acid at a rate of about 3 units. This rate was determined only by assessment of the intensity of the spots obtained by paper chromatography in butanol–acetic acid–water (40:9:20, by vol.), followed by treatment of the chromatogram with chlorine and *o*-tolidine (Reindel & Hoppe, 1954); the error is probably about 50 %. 5-Oxopyrrolidine-2-carboxylic acid was identified by comparison with a sample prepared from glutamic acid (Ellfolk & Synge, 1955), and by its hydrolysis to glutamic acid.

Breakdown of γ-glutamyl peptides

A solution (2 ml.) containing dialysed calf lens (1·54 ml.) and γ-L-glutamyl-L-α-amino-*n*-butyric acid (17 μmoles) was kept at 37° and pH 6·4. Paper electrophoresis showed the disappearance of the starting material and the appearance of only one ninhydrin-positive product, α-amino-*n*-butyric acid; no glutamic acid was formed. When the paper was treated with the chlorine–*o*-tolidine reagent (Reindel & Hoppe, 1954) the other product of the reaction was seen to be 5-oxopyrrolidine-2-carboxylic acid. The reaction is

$$H_2N\cdot CH(CO_2H)\cdot[CH_2]_2\cdot CO\cdot NH\cdot CHEt\cdot CO_2H \rightarrow HN\cdot CH(CO_2H)\cdot[CH_2]_2\cdot CO + H_2N\cdot CHEt\cdot CO_2H$$

The rate of breakdown of the dipeptide was 182 units; when the lens preparation was diluted 10 times the rate of breakdown was 18 units, so that the rate of the non-enzymic breakdown is negligible under these conditions.

A similar reaction occurred with γ-L-glutamylglycine; the rate of breakdown was 30 units.

Dipeptidase activity of lens extracts

Calf lens extracts catalysed the hydrolysis of L-α-amino-*n*-butyrylglycine; the rate of the reaction was not measured, but it took place more rapidly than any of the other reactions cited (cf. Kinoshita & Masurat, 1957). The dipeptidase of calf lens can be purified by the method which Binkley (1952, 1954) applied to kidney extracts; the unusual properties of this enzyme led Binkley to suggest that it was a polynucleotide, but subsequent work renders this unlikely (Semenza, 1957).

Synthesis of γ-glutamyl-α-amino-n-butyric acid

The substrates were L-glutamic acid (8 mM) and DL-α-amino-*n*-butyric acid (16 mM); the other constituents of the mixture were ATP (2·5 mM), 3-phosphoglyceric acid (22 mM), KCl (42 mM), $MgSO_4$ (10 mM), potassium phosphate buffer

(pH 6·4) (6 mM). The solution (10 ml.) containing dialysed calf lens (7·7 ml.) was kept at 37° and the pH was 6·3. The rate of decrease of the concentration of glutamic acid was 18 units, and the rate of increase of 5-oxopyrrolidine-2-carboxylic acid was about the same. The stationary state concentration of the dipeptide, attained after about 2 hr., was about 0·2 mM.

Synthesis of ophthalmic acid from γ-glutamyl-α-amino-n-butyric acid and glycine

The substrates were γ-L-glutamyl-α-amino-*n*-butyric acid (8 mM) and glycine (8 mM); the nature and concentration of the other constituents were the same as in the synthesis of ophthalmic acid from its component amino acids; dialysed calf lens was used. The rate of breakdown of the dipeptide was 180 units, and the rate of synthesis of ophthalmic acid was 63 units (Fig. 3); the formation of 5-oxopyrrolidine-2-carboxylic acid accounts for the difference between the two rates.

Synthesis of γ-glutamylalanylglycine and γ-glutamylthreonylglycine

Dialysed calf lens was used for these experiments and the incubation mixture resembled that used for the synthesis of ophthalmic acid from its component amino acids, except that α-amino-*n*-butyric acid was replaced by one of the following amino acids (8 or 16 mM, depending on whether the L- or the DL-form was used): alanine, threonine, valine, serine, homocysteine, homoserine, tryptophan, tyrosine, α-amino*iso*butyric acid, phenylalanine, methionine, β-alanine, isoleucine, leucine, lysine, proline, hydroxyproline, β-aminobutyric acid, aspartic acid, glutamine, cystine, asparagine, citrulline, histidine, ornithine, arginine, γ-aminobutyric acid, and the cysteine–*N*-ethylmaleimide adduct. Only the first two amino acids gave ninhydrin-positive products of the expected electrophoretic mobility. The products were isolated by elution from the electrophoresis papers, and the amino acid composition was determined by hydrolysis and quantitative paper chromatography. The product from alanine had the same R_F as norophthalmic acid (Waley, 1957), but it was contaminated with ophthalmic acid (not completely removed by dialysis and so present in the lens preparation) and the amino acids present in the hydrolysate, alanine, glutamic acid, glycine and α-amino-*n*-butyric acid were in the ratio 1·0:1·5:1·6:0·5. The product from threonine gave on hydrolysis threonine, glutamic acid and glycine, in the ratio 0·8:1·0:1·0. We conclude that both products are analogous to ophthalmic acid and glutathione. The rate of synthesis of γ-glutamylalanylglycine was about 1 unit, and that of γ-glutamylthreonylglycine about 0·5 unit; these are only approximate values, assessed by inspection of the spots.

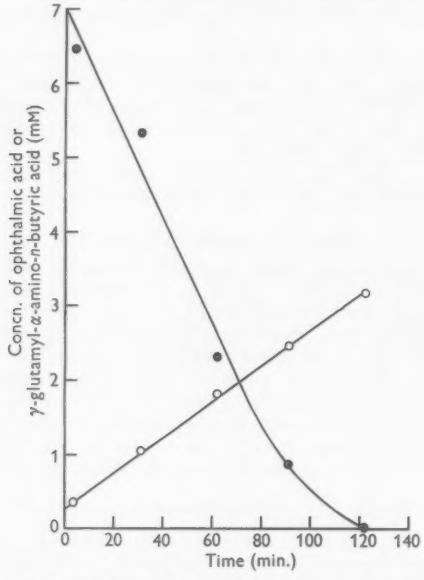

Fig. 3. Formation of ophthalmic acid (○) and decomposition of γ-glutamyl-α-amino-*n*-butyric acid (●), followed by paper electrophoresis.

Breakdown of ophthalmic acid in the liver

Fresh calf liver (50 g.) was macerated with 0·1 M-potassium phosphate (pH 7·4) (69 ml.), 0·16 M-KCl (28 ml.) and 0·16 M-$MgSO_4$ (3 ml.) at 4°, and a portion of the supernatant (4·0 ml.) used in a volume of 4·1 ml., containing 16 μmoles of the potassium salt of ophthalmic acid. The mixture was kept at 37°, at pH 7·3. The usual method of precipitating the proteins with ethanol was not adequate and the samples were subsequently acidified with 1% acetic acid to pH 5·2 and heated at 100° for 3 min. to complete removal of the proteins. Paper electrophoresis was used to measure the rate of breakdown of ophthalmic acid (42 units) and the rates of formation of glutamic acid (40 units) and neutral amino acids (92 units). As complete hydrolysis of ophthalmic acid liberates 2 mol. of neutral amino acids to 1 mol. of glutamic acid, the measured rates suggest a two-step mechanism, in which the second step is faster than the first:

Ophthalmic acid → glutamic acid + α-amino-*n*-butyrylglycine

α-Amino-*n*-butyrylglycine → α-amino-*n*-butyric acid + glycine

Estimation of ophthalmic acid in tissues

The ophthalmic acid content of tissues was investigated by a two-dimensional procedure: paper electrophoresis was followed by paper chromatography; this ensured a good separation of glutathione (which was oxidized to GSSG during the paper chromatography) and a better characterization of ophthalmic acid. Lenses were ground under ethanol (to give a final ethanol concentration of 70%, v/v), the protein precipitate spun down, the supernatant evaporated *in vacuo* and the residue dissolved in aq. 10% (v/v) propan-2-ol. A portion (5 μl.) was used for electrophoresis on a 25 cm. × 44 cm. sheet of Whatman no. 52 paper. Portions (5 μl.) of three standard solutions of ophthalmic acid were applied along the starting line for the chromatographic run in positions such that the ophthalmic acid was finally separated from any ninhydrin-positive compounds in the lens extract. Electrophoresis was carried out for 2 hr. (see Methods for further details). The dried paper was run in the second dimension for 24 hr., 72% (w/v) phenol–3% (w/v) NH_3 soln. being used as solvent. Glutathione was estimated (as GSSG) after paper chromatography in phenol–NH_3. The results, given in Table 2, are for calf and cow lenses; ophthalmic acid was not detected in rabbit or pigeon lenses, i.e. its concentration is less than 7 μmoles/100 g. of lens, and was possibly present at about this concentration in human lenses. Preliminary experiments failed to reveal ophthalmic acid in other tissues of the calf, but it is hoped to investigate these more thoroughly.

Reaction of pyruvic acid with amino compounds

Ninhydrin-positive substances with high electrophoretic mobility (migrating towards the anode on paper electrophoresis at pH 4) were frequently encountered during the synthesis of the peptide bond, when 3-phosphoglyceric acid is breaking down to pyruvic acid. Such acidic substances were then found to be formed quite rapidly from amino acids or peptides and pyruvic acid in neutral solution at room temperature. It has not been possible to examine these compounds in detail, as the extent of their formation varied unpredictably and they decompose on elution from dried electrophoresis papers, but it seems possible that these substances are the anils known to be formed from pyruvic acid and amines at pH 9 (Zuman, 1951).

DISCUSSION

Synthesis of ophthalmic acid: the overall reaction

Ophthalmic acid and glutathione are structurally similar and both synthesized by lens extracts. These observations suggest that the same enzyme may be responsible for the synthesis of both tripeptides. This possibility can be tested in the following way. If one enzyme (E) catalyses two reactions, as in the scheme shown below, then it can readily be shown that the rate of formation of the product (B) will be less when both the substrates A and C are present than when only one (A) is, unless the Michaelis constant of A is much less than that of C. The substrates may be pictured

$$A + E \rightleftarrows AE \rightarrow B + E$$
$$C + E \rightleftarrows CE \rightarrow D + E$$

as competing for the same active site on the enzyme, and so the greater the concentration of the enzyme–substrate compound CE the less the concentration of the compound AE, and hence the less the rate of formation of the product B. It was, in fact, found that the rate of synthesis of ophthalmic acid was depressed by the presence of cysteine (Fig. 2). There is, then, competition at one or both stages of the synthesis: cysteine and α-amino-*n*-butyric acid may compete in the first stage, or γ-glutamylcysteine and γ-glutamyl-α-amino-*n*-butyric acid may compete in the second. Thus at least one of the two enzymes involved in glutathione synthesis takes part in the synthesis of ophthalmic acid.

γ-Glutamyl lactamase in lens

Heating γ-linked peptides of glutamic acid in aqueous solution causes cyclization to 5-oxopyrrolidine-2-carboxylic acid (Le Quesne & Young, 1952) and the same reaction is catalysed by an enzyme (γ-glutamyl lactamase) in liver extracts (Hanes & Connell, 1956). Lens extracts were found to effect the conversion of γ-glutamyl-α-amino-*n*-butyric acid (γ-GluAB) into 5-oxopyrrolidine-2-carboxylic acid (PCA) and α-amino-*n*-butyric acid (AB):

$$\gamma\text{-GluAB} \rightarrow PCA + AB. \quad (1)$$

Table 2. *Concentration of ophthalmic acid in lens*

Lens	Wt. of lens used (g.)	Ophthalmic acid (μmoles/100 g. of lens)	Glutathione (μmoles/100 g. of lens)	Ratio of concn. of glutathione to concn. of ophthalmic acid
Cow	10·9	118	1220	10·3
Calf	10·0	121	1060	8·8
Calf	7·8	86	820	9·5

This reaction is apparently confined to the dipeptide; glutamic acid and ophthalmic acid are unaffected on incubation with lens extracts. The relatively rapid decomposition of γ-glutamyl-α-amino-*n*-butyric acid necessarily affects the overall rate of ophthalmic acid synthesis in crude lens extracts.

The two stages of the synthesis of ophthalmic acid

The view that the first stage of the synthesis of ophthalmic acid is the formation of γ-glutamyl-α-amino-*n*-butyric acid is confirmed by the observation that this dipeptide is indeed formed from the amino acids (and ATP) by lens extracts:

$$\text{Glu} + \text{AB} \rightarrow \gamma\text{-GluAB}. \qquad (2)$$

Only a low concentration (0·2 mM) of the dipeptide was built up, as it breaks down rapidly by the reaction (1) shown above. The net effect of reactions (1) and (2) is the conversion of glutamic acid into 5-oxopyrrolidine-2-carboxylic acid.

The second stage of synthesis of ophthalmic acid is the reaction of γ-glutamyl-α-amino-*n*-butyric acid with glycine (reaction 3):

$$\gamma\text{-GluAB} + \text{Gly} \rightarrow \gamma\text{-GluABGly}. \qquad (3)$$

The presence of γ-glutamyl lactamase in lens extracts results in the dipeptide decomposing more rapidly than ophthalmic acid is formed (Fig. 3). The rate of synthesis of ophthalmic acid in this experiment was about five times as great as that from the three amino acids, as the initial concentration of the dipeptide in the first case was much higher than the stationary-state concentration reached in the second.

These results show that the synthesis of ophthalmic acid in lens extracts proceeds by the same two stages as that of glutathione in liver extracts. The formation of 5-oxopyrrolidine-2-carboxylic acid limits the synthesis of ophthalmic acid; it is not known to what extent this also applies to the synthesis of glutathione.

Synthesis and occurrence of other tripeptides

Glutathione and ophthalmic acid are not the only tripeptides occurring in calf lens; norophthalmic acid (γ-glutamylalanylglycine) has been detected (Waley, 1957) and, as hydrolysis of an acidic fraction liberates threonine (Waley, 1956), γ-glutamylthreonylglycine may also be present. The question then arises whether lens extracts will catalyse the synthesis of several peptides of the type γ-Glu(A).Gly, where A is the residue of an amino acid other than cysteine or α-amino-*n*-butyric acid. Out of a wide range of amino acids tested, only alanine and threonine gave rise to tripeptides; the rate of formation of both these peptides was considerably lower than that of ophthalmic acid. Had any of the other amino acids reacted still more slowly, the resulting tripeptide would have been difficult to detect; in fact, there was some evidence that traces of the tripeptide containing valine were formed, but the rate of synthesis was too low to be sure about this. Similarly, on some occasions hydrolysis of the acid fractions of lens extracts released traces of valine (Waley, 1956). There seems, then, to be a parallel between the ability of calf lens extracts to synthesize tripeptides of this type and the occurrence of these tripeptides in the lens. This suggests that the steady-state concentration of these compounds *in vivo* may be governed mainly by their rate of synthesis. Some confirmation of this (although in pathological conditions) is provided by the observation that the decrease in the concentration of glutathione in cataract in rabbit lens is directly proportional to the decrease in the rate of synthesis of the peptide by extracts of these lenses (Daisley, 1955). No breakdown was detectable when either glutathione or ophthalmic acid was incubated with calf lens or rabbit lens extracts, i.e. no catabolic reactions are demonstrable.

The simple idea that the concentrations of these peptides *in vivo* are governed by their rate of synthesis, and that these can be gauged by experiments with tissue extracts, seems to hold well enough for calf lens, but unfortunately lacks generality. No ophthalmic acid could be detected in rabbit lens, and yet the rate of synthesis in rabbit lens extracts is no less than that in calf lens extracts (Table 1). It is, of course, possible that the concentration of α-amino-*n*-butyric acid may be much greater in calf lens than in rabbit lens, but there is no evidence to settle this point.

Metabolism of ophthalmic acid in the liver

A natural extension of the work described above on lens was an investigation whether ophthalmic acid was present, built up or broken down, in another tissue. Liver was chosen, as it contains much glutathione, and liver extracts synthesize glutathione (Snoke & Bloch, 1954). Ophthalmic acid was not detected in calf liver, nor was its synthesis by liver extracts demonstrable, but the rate of breakdown of ophthalmic acid in the extracts was quite high—about four times that of the synthesis by lens extracts. So this may well account for the failure to show net synthesis by liver extracts. Moreover, the breakdown was zero-order, and if it is still zero-order when ophthalmic acid is present at a concentration of 10 μmoles/100 g. of tissue, the half-life for the decomposition of ophthalmic acid in the extracts would only be about 1 min., and thus its presence in the liver would not be detected. The decomposition of ophthalmic acid in the liver extracts gave glutamic acid; little

or no 5-oxopyrrolidine-2-carboxylic acid was formed. The direct hydrolysis of the γ-glutamyl peptide bond has also been observed by Woodward & Reinhart (1942), who found that glutathione was hydrolysed to glutamic acid by kidney extracts in the pH range 5–7; in more alkaline solution, however, formation of 5-oxopyrrolidine-2-carboxylic acid was observed. Cyclization to the oxopyrrolidine occurs preferentially in alkaline solution because only the uncharged amino group is nucleophilic; formation of intermolecular amide, too, is favoured (at the expense of hydrolysis) by raising the pH (Fruton, 1955–56).

SUMMARY

1. Lens extracts catalyse the synthesis of the tripeptide, ophthalmic acid, from its constituent amino acids and adenosine triphosphate. The synthesis proceeds through the intermediate formation of γ-glutamyl-α-amino-*n*-butyric acid.

2. Either (or both) of the enzymes of glutathione synthesis are also involved in the synthesis of ophthalmic acid.

3. Analogous tripeptides are synthesized when α-amino-*n*-butyric acid is replaced by alanine or threonine.

4. Liver extracts catalyse the hydrolysis of ophthalmic acid to its three amino acids.

5. Rabbit lens extracts contain little, if any, ophthalmic acid, but catalyse its synthesis just as effectively as do calf lens extracts.

We should like to thank our colleagues at this Laboratory for advice and encouragement, Miss Blackwell and Mrs Davies for the collection of calves' eyes, the staffs of the Oxford Co-operative and Eastwyck Farm Slaughterhouses for their co-operation, the National Council to Combat Blindness, Inc., New York, for a Fight for Sight Grant for the purchase of chromatographic equipment, and The Distillers' Co. Ltd. for a gift of glutathione. One of us (E.E.C.) is in receipt of a Thornton Bursary.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **371st Meeting** *of the Biochemical Society was held in the Department of Physiology, The Medical School, Birmingham* 15, *on Friday,* 28 *March* 1958, *starting at* 11 *a.m. when the following papers were read:*

COMMUNICATIONS

Pyrimidine Biosynthesis in *Escherichia coli*. By G. W. Crosbie. (*Department of Biochemistry, University of Glasgow*)

Information has been sought, with the aid of labelled precursors, as to the mechanism of thymine ring biosynthesis in cells of *Escherichia coli* growing exponentially in a simple synthetic medium.

A study of the incorporation of [^{14}C]bicarbonate, [^{14}C]formate, [β-^{14}C]serine and [2-^{14}C]glycine into the nucleic acids and proteins of a wild-type strain, *Esch. coli* N.C.T.C. 5928 and of a uracil-less mutant, *Esch. coli* M 63–86 (kindly provided by Dr B. D. Davis), has indicated:

(*a*) That formate does not function as a thymine methyl group precursor although incorporation into positions 2 and 8 of the purine ring occurs. These findings have been confirmed by the following degradation studies: (i) guanine to glycine and 4-guanidinoglyoxaline (Hunter, 1936); (ii) adenine to glycine and 4-amino-5-glyoxaline carboxamidine (Cavalieri, Tinker & Brown, 1949); and (iii) thymine to iodoform (Elwyn & Sprinson, 1954).

(*b*) That [2-^{14}C]glycine and [β-^{14}C]serine function as thymine methyl group precursors.

(*c*) That in the absence of extracellular supplements, the thymine methyl group is quantitatively derived from the β-carbon of serine by a pathway which does not involve glycine.

(*d*) That the extent of utilization of [2-^{14}C]-glycine for 1-C unit synthesis is dependent on its extracellular concentration (cf. Koch, 1955).

(*e*) That the pathway of incorporation of [2-^{14}C]-glycine into the thymine methyl group does not involve serine.

The incorporation of [β-^{14}C]serine into the DNA-thymine of a methionine-less mutant of *Esch. coli*, CW 194 (kindly provided by Dr J. Ceithaml), has indicated in confirmation of the work of Green & Cohen (1957) that the methionine methyl group is not a thymine precursor.

The results of this investigation are consistent with the role of hydroxymethyltetrahydrofolic acid as the more immediate 1-carbon donor in thymine ring biosynthesis.

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Elwyn, D. & Sprinson, D. B. (1954). *J. biol. Chem.* **207**, 467.
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Biosynthesis of Thymidylic Acid. By G. D. Birnie and G. W. Crosbie. (*Department of Biochemistry, University of Glasgow*)

Blakley (1957) and Phear & Greenberg (1957) have recently reported the synthesis by soluble mammalian enzymes of thymidine from deoxyuridine and [β-^{14}C]serine and of thymidylic acid from deoxyuridine and [^{14}C]formaldehyde. Friedkin & Kornberg (1956) and Flaks & Cohen (1957) have also reported the synthesis of thymidylic acid from deoxyuridylic acid by cell-free extracts of *Escherichia coli*. The latter authors have, in addition, prepared 5-hydroxymethyldeoxycytidylic acid enzymically from deoxycytidylic acid.

In the present work a cell-free extract of a serine-less mutant of *Esch. coli*, PA/15, was prepared by alumina grinding followed by extraction with Tris buffer (0·1 M, pH 7·4). The extract, which effects a tetrahydrofolic acid-dependent serine–glycine interconversion with simultaneous formation of hydroxymethyltetrahydrofolic acid, was incubated under N_2 with deoxyuridine (0·01 M), [β-^{14}C]serine (0·002 M, 1 μc/ml.), $MgSO_4$ (0·01 M), and substrate amounts of ATP, TPNH and tetrahydrofolic acid. Thymidylic acid and thymidine together with a trace of thymine have been detected by chromatographic examination of the incubation mixture. The thymine derivatives have been isolated with the aid of carriers and the identity of the labelled

products confirmed by autoradiography following extensive chromatographic and ionophoretic separations.

The structures of two labelled products with chromatographic properties similar to 5-hydroxymethyldeoxyuridylic acid and the corresponding nucleoside are under investigation.

Replacement of TPNH by DPNH greatly reduced the yield of thymine derivatives. No labelled pyrimidine derivatives were formed in the presence of phosphate buffer due to the powerful nucleoside phosphorylase activity of the enzyme preparations used.

We are indebted to Dr D. D. Woods for a gift of *Esch. coli* PA/15. One of us (G. D. B.) wishes to acknowledge the receipt of a grant from the Medical Research Council.

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Purification of the Nicotinic Acid Hydroxylase System. By A. L. Hunt.* (*Department of Biochemistry and Medical Research Council Unit for Research in Cell Metabolism, University of Oxford*)

Cell-free extracts of nicotinic acid-adapted *Pseudomonas fluorescens* KB1 oxidize nicotinic acid to 6-hydroxynicotinic acid with concomitant uptake of oxygen. The oxygen added in the 6-carbon position is derived from water (Hunt, Hughes & Lowenstein, 1957, 1958), suggesting that hydroxylation involves addition of water to the ring structure, followed by dehydrogenation coupled to molecular oxygen.

The enzyme preparation utilizes electron acceptors other than oxygen, such as ferricyanide, 2:6-dichlorphenolindophenol, methylene blue, tetrazolium compounds, and phenazine methosulphate. Enzyme activity may conveniently be assayed by following the reduction of ferricyanide spectrophotometrically.

Centrifugal fractionation of extracts, prepared as previously described, indicated that activity resided entirely in insoluble particulate fractions of the cell. The greater part occurred in the pink layer which sedimented above smaller layers of damaged cells at 60 000 *g* for 60 min. Under the electron microscope this fraction appeared to consist mainly of cell walls with protoplast membrane and some residual electron dense material attached, together with small amounts of fragments of the order 50–100 mμ in diameter. The specific activity of the latter, separated by differential centrifugation at 10 000 *g*, was low compared with the bulk of the fraction.

For the purification of the enzyme the washed pink fraction was homogenized in a glass Potter–Elvehjem homogenizer at 0°, with the addition of 1 mg. sodium deoxycholate/12·5 mg. protein, in 0·05 M-potassium phosphate pH 7·15. The homogenate was then centrifuged at 100 000 *g* for 60 min. This treatment released most of the enzyme activity into the supernatant, together with considerable flavin and cytochrome pigment. The bulk of this activity precipitated between 15 and 35% saturated ammonium sulphate. The bright pink precipitate so obtained dissolved readily in 0·01 M-phosphate buffer pH 7·15, yielding a clear brick-red solution, exhibiting a Tyndall effect.

This preparation oxidized nicotinic acid rapidly with ferricyanide and with molecular oxygen. Addition of nicotinate in the absence of electron acceptors produced a marked change in the spectrum of the solution with maxima appearing at 560, 553, 550, 530, 524, 425–28 mμ, and a depression at 460 mμ, enhanced by the addition of a trace of solid potassium cyanide, and still further by dithionite. Reduced pyridine nucleotide was not oxidized. The preparation exhibited very active cytochrome-*c*-peroxidase activity (Lenhoff & Kaplan, 1956). The ratio of reduced cytochrome to flavin in the presence of nicotinate and cyanide was 3:2, and with dithionite 2:3. Based on the latter, the concentration of total haem and flavin averaged $1{\cdot}2 \times 10^{-3}$ μmole/mg. $N \times 6{\cdot}25$ and $1{\cdot}8 \times 10^{-3}$ μmole/mg. $N \times 6{\cdot}25$ respectively. Overall enzyme purification based on ferricyanide reduction averaged 60-fold, compared with the whole homogenate.

The author is indebted to Dr D. E. Hughes for assistance in growing bacteria, Dr G. Meek for electron micrographs, and Dr J. M. Lowenstein for helpful discussions.

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The Distribution of ^{15}N fixed by Root Nodules of *Alnus glutinosa*. By G. Bond, I. Gardner and G. Leaf. (*Departments of Botany and Biochemistry, University of Glasgow*)

Extracts of *Alnus* nodules which had been exposed to $^{15}N_2$ were fractionated and the distribution of ^{15}N among amino acids and ammonia was investigated. The highest atom % excess of ^{15}N was found in glutamic acid and the next highest in citrulline or aspartic acid. Ammonia always contained less ^{15}N than these compounds, and arginine contained very little ^{15}N. When citrulline was degraded to ammonia and ornithine it was found that the ammonia liberated was even richer in ^{15}N than was glutamic acid. The significance of these findings will be discussed.

Improved Methods for the Paper Chromatography of Organic Acids. By G. J. Lawson and R. D. Hartley. (*Department of Mining, Birmingham University*)

The separation of organic acids by paper chromatography is subject to two principal difficulties, namely, the streaking produced by partial ionization of the acids, and the relatively poor differentiation produced on spraying with acid-base indicators.

Ionization is usually controlled by using either acid or basic solvent mixtures, but most systems fail adequately to prevent formation of streaks by oxalic acid, a frequent component of acid mixtures from chemical degradations. Oxalic acid has been found to move as a discrete spot on Whatman No. 1 paper when irrigated by an upward-flow method (Bray, Thorpe & White, 1950), using either a mixture of ethyl acetate (2 vol.), glacial acetic acid (1 vol.) and water (1 vol.), or a mixture of ethyl formate (2 vol.), 98 % formic acid (1 vol.) and water (1 vol.). In the former solvent R_F values vary somewhat with the amount of acid placed on the chromatogram but this effect is not found with the formate mixture. This latter solvent has the added advantage that R_F values for oxalic and some other simple acids are high, so that these do not interfere with more complex, slower-moving acids.

Acid-base indicators give best results on paper chromatograms when their amount and distribution are carefully controlled. This is difficult when using sprays, but may readily be achieved by adding to the solvent an indicator having a suitable pH range and an R_F value of approximately 1·0.

For the acid solvents mentioned above methyl red in a concentration of 0·008 % (w/v) is suitable, but in formate-containing solvents even better results can be obtained using bromophenol blue (0·015%, w/v). Sodium acetate or sodium formate (0·05 %, w/v) is incorporated to suppress the formation of 'acid bands' on the chromatograms. After irrigation the chromatograms are dried in air, when the acids are revealed as red spots on a pink background. There is no tendency to fade, in fact the chromatograms improve with keeping. The solvent boundary is marked automatically.

For use with a routine basic solvent (ethanol 70 %, water 26 %, 0·880 ammonia 4 % by volume), phenol violet and thymol blue are suitable as indicators. With the alkaline solvent the acid spots are visible while the solvent is actually flowing, so that rapid comparisons of samples may be made.

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Oxygen Tension and the Inhibition of Nitrite Oxidation by *Nitrobacter*. By W. D. Butt and H. Lees. (*Department of Biological Chemistry, University of Aberdeen*)

The cyanate ion is normally a powerful inhibitor of the aerobic oxidation of nitrite to nitrate carried out by *Nitrobacter* (Lees & Simpson, 1957). We have now observed that when high densities of *Nitrobacter* cells are used, cyanate stimulates nitrite oxidation. The reversal of cyanate activity is apparently due to the fact that high cell densities, with their high O_2 demand, cause a marked lowering of O_2 tension in the cell-suspension. A similar reversal of cyanate activity can be brought about at lower cell densities if the partial pressure of O_2 in the atmosphere in contact with the cell-suspension is reduced. A reversible activity towards nitrite oxidation similar to that of cyanate (inhibition at normal O_2 tensions, stimulation at low O_2 tensions) is shown by arsenite and nitrate but not by phosphate, chloride, or sulphate. We have also found that, as the O_2 tension is reduced, nitrite becomes progressively more toxic to its own oxidation and as a consequence the optimum nitrite concentration for nitrite oxidation by *Nitrobacter* progressively falls.

We suggest as an explanation of these results that nitrate, arsenite and cyanate ions, being somewhat similar to the nitrite ion in size and structure, compete with it in gaining access to the nitrite-oxidizing mechanisms of the cell. At low O_2 tensions, where nitrite becomes toxic to its own oxidation, the resulting effective reduction of nitrite concentration in the vicinity of the oxidizing mechanisms results in a stimulation of nitrite oxidation. At normal O_2 tensions the same reduction of nitrite concentration in the vicinity of the oxidizing mechanisms results in an inhibition of nitrite oxidation.

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The Enzymic Degradation of 'Raw' Starch Granules. By J. R. Turvey and R. C. Hughes. (*Department of Chemistry, University College of North Wales, Bangor*)

Intact starch granules, in contrast with starch which has been brought into solution, are strongly resistant to the usual preparations of amylases from animal and microbial sources (Balls & Schwimmer, 1944; Schwimmer, 1945) or plant sources.

Sandstedt and co-workers (Blish, Sandstedt & Mecham, 1937; Sandstedt & Gates, 1954) isolated a 'raw starch factor' from germinated cereals which slowly attacked a variety of granular starches. There was evidence that this factor was not identical with α-amylase.

We have now carried out a preliminary examination of the action of potato juice on raw starch granules. By precipitation of cold aqueous extracts of sprouted potatoes with ammonium sulphate we have obtained protein fractions which slowly attack potato starch grains, as evidenced by microscopic examination (Baker & Hobson, 1952). Similar extracts of ungerminated wheat had no action on raw starch until mixed with potato extract. The degradation produced by the mixture was easily followed by measurement of the reducing power of the supernatant.

The presence of an activator as well as an inhibitor of this reaction has been demonstrated in potato juice. The activator, which is present in ashed potato juice, is probably the phosphate ion. Thus we have found that inorganic phosphate activates the raw starch factor and that glucose 1-phosphate (tentatively identified by paper chromatography) is one of the main products of degradation. The inhibitor is dialysable, loses its inhibitory power on being boiled in water for 5 min. and is soluble in ether to a certain extent.

Further tests have indicated that phosphorylase prepared from potato by the method of Hobson, Whelan & Peat (1950) or by adsorption on amylose (Baum & Gilbert, 1953) attacks starch grains in the presence of mineral phosphate but not in its absence. The action is diminished in the presence of the inhibitor mentioned above, which may be related to the inhibitor of phosphorylase present in the potato (Schwimmer, 1957). Inhibition of the traces of amylase impurity present in the phosphorylase preparations by mercuric ion (Bailey, Thomas & Whelan, 1951) did not affect the ability of the enzymes to attack raw starch.

There is thus some indication that the 'raw starch factor' is phosphorylase. This possibility is being further explored.

We thank Professor S. Peat, F.R.S., for his advice and encouragement.

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***N*-Acetylation and Mercapturic Acid Formation in the Rabbit, Rat and Guinea pig *in vivo* and in their tissues *in vitro*.** By H. G. Bray, T. J. Franklin and Sybil P. James. (*Department of Physiology, Medical School, University of Birmingham*)

Experiments on the possible role of glutathione and glutathionase in mercapturic acid formation have already been reported (Barnes & James, 1957; Bray & Franklin, 1957). The liver of guinea pig is a richer source of glutathionase than is that of either the rabbit or the rat, so that the guinea pig might be expected to synthesize mercapturic acids in amounts comparable with those formed by the rabbit and the rat. Corner & Young (1954), however, found that the guinea pig formed much

less mercapturic acid from naphthalene than did the rabbit and the rat. It seemed of interest, therefore, to compare the amounts of mercapturic acid formed by the three species *in vivo* and by their tissues *in vitro* from different types of mercapturic acid precursors. The compounds studied include chlorobenzene, benzyl chloride, 3:4-dichloronitrobenzene and 2:3:4:6-tetrachloronitrobenzene which form mercapturic acids readily in the rabbit (e.g. Smith, Spencer & Williams, 1950; Stekol, 1938; Bray, James & Thorpe, 1957; Betts, James & Thorpe, 1955). The results show that *in vivo* the rat closely resembles the rabbit in forming appreciable amounts (not less than 18% of the dose) of mercapturic acid from the compounds mentioned, whereas the guinea pig forms very little. When *S*-substituted L-cysteines formed from the halogenocompounds were administered to the three species mercapturic acid was readily formed by the rabbit and the rat, but in most cases less readily by the guinea pig.

When acetylation of some amino compounds by liver slices of the three species was examined it was found that rabbit and rat slices readily acetylated sulphanilamide, D-glucosamine and *S*-(*p*-chlorobenzyl)-L-cysteine. Liver slices of the guinea pig readily acetylated sulphanilamide but neither D-glucosamine nor *S*-(*p*-chlorobenzyl)-L-cysteine was acetylated to a significant extent. Under the conditions used no acetylation of any of the amino compounds was detected in kidney slices. The results both *in vivo* and *in vitro* suggest that one factor in the failure of the guinea pig to form large amounts of mercapturic acid may be an inefficient mechanism for the acetylation of aliphatic amino groups in the liver.

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Sorbitol in Animal Tissues. By H. G. Britton (introduced by A. St G. Huggett). (*Department of Physiology, St Mary's Hospital Medical School, London, W. 2*)

Evidence has been obtained for the presence of sorbitol *post mortem* in ram seminal vesicle, rat coagulating gland and foetal whale blood and, in traces, in fresh foetal sheep blood. None was found in fresh new-born calf blood.

Identification was effected by the behaviour of the material on paper chromatography in several solvent systems, by its slow reaction with alkaline silver nitrate, by the colour developed with boric acid indicator reagent, by its rapid reaction with periodate and by its lack of adsorption on basic ion exchange resins. Comparison with the other isomeric hexitols made a provisional identification of sorbitol possible. Further identification of ram and whale material was effected by single crystal X-ray diffraction on the hexa-acetate (Dr T. Lomer, Physics Department, St Mary's Hospital Medical School) and by reaction with sorbitol dehydrogenase.

When ram seminal vesicle was frozen rapidly in liquid nitrogen only a trace of sorbitol (in relation to fructose) was found; an appreciable increase occurred with a delay of 5 min. before freezing, and with a delay of several hours the quantity of sorbitol was of the same order as the fructose content. The rise in sorbitol could possibly be due to reduction of fructose, reduction of glucose or liberation from a conjugate. Hydrolysis with hydrochloric acid, however, gave no increase in sorbitol. One specimen of foetal sheep blood from a grossly anoxic foetus appeared to contain increased quantities of sorbitol, and it is suggested that (1) the larger quantity of sorbitol in foetal whale blood was probably a post-mortem artifact, and (2) fructose may possibly act as a physiological hydrogen acceptor during anoxia.

These results confirm the tentative identification of sorbitol made by Hers (1957) in *post mortem* specimens of ram seminal vesicle. With regard to the presence of traces of sorbitol in sheep foetal blood, similar observations have been made by M. W. Neil (personal communication).

Experiments with radioactive sugars confirm (Hers, 1957) that sorbitol is converted to fructose by ram seminal vesicle but not vice versa. In view of the above, however, it is suggested that these results may indicate that the cells are permeable to sorbitol but not fructose rather than indicating the direction of the reaction.

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Scintillation Counting of Tritium in Solid Samples. By W. G. DUNCOMBE. (*The Wellcome Research Laboratories, Beckenham, Kent*)

Methods in general use for the measurement of the very low energy β-radiation from tritium include gas counting, liquid scintillation counting and windowless gas-flow counting of solid samples. In the first two methods the sample cannot be recovered, and this may be true also of the last method. Difficulties in counting sometimes occur due to the build-up of static charges on the sample, and devices to make the sample conducting may then be required, such as mixing it with powdered graphite (Banks, Crawhall & Smyth, 1956).

A non-destructive method for counting solid samples would be of use in a number of ways, including a preliminary screening of samples before the more refined but laborious gas counting.

Experiments have shown that scintillation counting of solid tritium-labelled samples deposited in planchettes is quite feasible with comparatively simple apparatus. Commercial electronic apparatus (Isotope Developments, Ltd.) was used, together with a single, unselected photomultiplier tube (EMI 6097), cooled with solid CO_2/ethanol, to the window of which was attached a thin disk of plastic phosphor. The photomultiplier tube unit was mounted in a lightproof housing fitted with an inner sliding door and an outer fabric sleeve, so that samples could be changed in complete darkness. With an EHT voltage of 1000, maximum amplification, and discriminator set at 12·5 v, a background of 300–500 counts/minute was obtained. With the planchette containing the active sample placed against the phosphor reproducible counting rates were obtained, though the efficiency was low. This was greatly increased by eliminating the absorption of β-particles by the air between the sample and phosphor. The sample was placed in a small demountable chamber, one end of which consisted of the phosphor disk, which was then evacuated. By this means the efficiency of counting an infinitely thin sample of tritiated stearic acid was raised from about 1 to 10 %.

The cooled photomultiplier tube arrangement is also suitable for homogeneous liquid scintillation counting of tritium. An efficiency of about 50 % was obtained with 10 μg. of tritiated stearic acid dissolved in 5 ml. of a *p*-terphenyl/toluene phosphor.

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The Production of Secondary Potassium Depletion, Hypercalcaemia and Nephrocalcinosis by Magnesium Deficiency. By I. MACINTYRE and D. DAVIDSSON. (*Postgraduate Medical School, Ducane Road, London, W.* 12)

A group of fifteen female rats (mean initial weight 100 g.) were fed a diet containing 0·5 mM Mg/kg. A control group were fed the same diet with added Mg. Rats from each group were killed at intervals throughout the experiment, which lasted for 70 days. The characteristic vasodilatation of Mg deficiency described by Kruse, Orent & McCollum (1932) occurred after 12 days and lasted for a further 10 days. The following changes were found in tissue (the results are expressed as m-equiv./kg. dry fat-free solids $\pm$ S.E.M.).

Muscle. There was a progressive fall in Mg reaching 78 % of the initial value after 64 days. This was accompanied by an equal fall in K. There was a highly significant correlation ($r = 0{\cdot}93$, $P < 0{\cdot}01$) between muscle Mg and K. The deficient group showed a mean increase of 51 ± 8 and 35 ± 5 in Na and Cl respectively. The difference in collagen content of the two groups was negligible.

Kidney. The deficient animals showed a marked increase in Ca to $52{\cdot}5 \pm 13$ from the control value of $12{\cdot}4 \pm 0{\cdot}5$. This only occurred in those animals with hypercalcaemia. Mg and K concentrations were unchanged.

Liver and brain. The Mg, K and Ca concentrations did not differ in the two groups.

Plasma. The deficient group showed a slight increase in Na, a marked fall in Mg, together with a rise in Ca (deficient group 3·9–8·0, $6{\cdot}4 \pm 0{\cdot}3$, control 4·9–6·0, $5{\cdot}3 \pm 0{\cdot}1$ m-equiv. Ca/kg. plasma).

Bone. At 3 weeks the Mg concentration in the deficient group was approximately half that in the controls (cf. Watchorn & McCance, 1937).

Vitale, Nakamura & Hegsted (1957) showed that mitochondria from Mg-deficient rats were abnormal. *In vitro*, Mg directly or indirectly aids in the maintenance of mitochondrial structure (Tapley, 1956; Baltscheffsky, 1957) upon the integrity of which the ion gradients found by Macfarlane & Spencer (1953) and Bartley & Davies (1954) depend. It is suggested that the K loss may have been due to an effect of Mg deficiency on to ion transport *in vivo*.

While it is possible that the hypercalcaemia is due to hyperparathyroidism, no direct evidence of this was obtained.

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The Behaviour of Glycerylphosphoryl Inositol and Related Phosphates on Ion-exchange Columns. By J. N. Hawthorne and G. Hübscher. (*Department of Medical Biochemistry and Pharmacology, University of Birmingham*)

Dawson (1954) has demonstrated interesting differences between diphosphoinositide and other brain phospholipids in their rate of incorporation of inorganic ^{32}P. Other workers in this field (Hokin & Hokin, 1955; McMurray, Strickland, Berry & Rossiter, 1957) have also made use of Dawson's method, which depends on mild alkaline hydrolysis of the phospholipids and separation of the resulting phosphate diesters by two-dimensional paper chromatography. Certain phosphoric acid esters found on these chromatograms remain unidentified and the origin of the highly labelled glycerophosphate is still uncertain (Dawson, 1954). In addition, the hydrolysis products of the inositol phosphatides in brain and other tissues are not well characterized, nor do they separate easily on the chromatograms.

An attempt has been made to extend Dawson's method by the use of basic ion-exchange resins rather than paper chromatography for the final separation of the esters. Preparations of brain phosphatidyl serine and liver monophosphoinositide have been studied in detail, but the method has not yet been applied to lipids obtained directly from tissues. Columns of Nalcite SAR (200 to 400-mesh, column dimensions 1×12 cm.) or Dowex 1 (2% cross-linked, 100 to 200-mesh, column dimensions 1×20 cm.) have been used for quantities of hydrolysate containing 8–12 mg. P. Using larger amounts of hydrolysate, the method may readily be adapted to the isolation of the pure phosphates in solid form.

Gradient elution with formic acid-ammonium formate mixtures gave poor separations, but in sodium tetraborate–ammonium formate mixtures the following compounds could be separated on one column: glycerophosphoryl choline (not adsorbed); glycerylphosphoryl serine (0·005B, 0·09F); glycerylphosphoryl inositol (0·005B, 0·06F); inositol monophosphoric acid, glycerophosphoric acid and orthophosphoric acid (0·005B, 0·15F). The figures in brackets indicate the molar concentrations of sodium tetraborate (B) and ammonium formate (F) required for elution of the various substances from a Nalcite SAR column. The identity of the phosphates was established, after removal of borate, by hydrolysis and chromatography of the products as previously described (Hübscher & Hawthorne, 1957).

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Acetylation of Sulphanilamide by Enzymes of Pigeon Liver *in vitro*. By Dorothy M. Fraser. (*Department of Physiology, Medical School, University of Birmingham*)

The mechanism of acetylation of aromatic amino compounds by liver appears to comprise two reactions. First, acetic acid is activated by the formation of a thio-ester with co-enzyme A (Co A) (catalysed by enzyme *A*), and secondly the acetyl group is transferred from Co A to an acceptor, e.g. sulphanilamide (enzyme *B*).

The formation of acetylsulphanilamide has been shown to occur *in vivo* in most common mammals and in birds. *In vitro* great difficulty has been found in preparing a soluble enzyme system from mammalian tissues. For this reason homogenates and acetone-dried preparations of pigeon liver are the best enzyme source (Kaplan & Lipmann, 1948).

Within limits, acetylation of the amine by crude acetone-dried preparations is roughly proportional to the amount of Co A present. Maximum utilization, however, never exceeds 70% of the sulphanilamide. If the enzyme is purified by further acetone precipitation or is prepared at temperatures above 0° it is inactive when supplied with Co A. When acetyl Co A replaces Co A, sulphanilamide is rapidly acetylated in amounts equivalent to the acetyl Co A present. If acetyl Co A is in excess the reaction proceeds until all the sulphanilamide is utilized. Thus enzyme *A* appears to be the less stable of the two. Preliminary results indicate that purified extracts of rat liver also acetylate the amine when acetyl Co A replaces Co A. Enzyme *B* is not only inactive in presence of Co A but is inhibited by it even in presence of acetyl Co A. Enzyme *A* has been prepared from pigeon liver by the method of Chou & Lipmann (1952) and when mixed with purified enzyme *B*, sulphanilamide is acetylated in presence of Co A.

Homogenates of pigeon liver in a medium of polyvinyl pyrrolidone and sucrose have been fractionated in a high-speed centrifuge. Enzyme *A* is located in the mitochondria and enzyme *B* in the fraction containing microsomes and soluble enzymes. The enzymes *A* and *B* thus obtained behave similarly to those from acetone-dried preparations. The activity is fairly constant from pH 7 to 8·5 with a probable optimum at pH 8·0.

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Kinetics of Acetylation of Sulphapyrazine in the Rabbit. By K. White. (*Department of Physiology, Medical School, University of Birmingham*)

The form of the excretion curves of sulphapyrazine and acetylsulphapyrazine after administration of sulphapyrazine to the rabbit has been investigated by the procedure previously used in a study of the kinetic aspects of metabolism of benzoic acid and other compounds (Bray, Thorpe & White, 1951). It was found that excretion of both the free and acetylated forms could be well represented by exponential curves. As acetylsulphapyrazine was found to accumulate slowly in the blood, it is not possible to equate the rate of excretion and rate of conjugation as in other conjugation reactions previously investigated. The first-order velocity constant which can be derived from the exponential excretion curve of the acetylsulphapyrazine is therefore a function of the processes of formation and of excretion. The acetylsulphapyrazine excretion curves were analysed carefully in order to detect any traces of linearity, but no such indication of a limiting rate was found. In agreement with this, no significant variation in the proportion of the dose acetylated was found over a range of dose level of 0·05–1·2 g. There was some variation in the proportion acetylated by different rabbits, which appears to be determined chiefly by variations in the velocity constant for excretion of free sulphapyrazine.

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Observations on Spermine Oxidase. By H. Blaschko and Rosemary Hawes. (*Department of Pharmacology, University of Oxford*)

The enzyme spermine oxidase was first found in the blood plasma of the sheep (Hirsch, 1953); it also occurs in the plasma of the ox (Tabor, Tabor & Rosenthal, 1954) and the goat (Bergeret & Blaschko, 1957).

A close relationship between the rumen and the presence of spermine oxidase is made likely by these observations:

(1) The sera of a number of mammalian species have been examined; none of these had a significant action upon spermine, with the exception of the serum of another ruminant, *Camelus dromedarius* L. The camel serum oxidized spermine and spermidine at similar rates; benzylamine was oxidized more slowly.

(2) Spermine oxidase is absent or almost absent from the serum of newborn kids, in which the rumen is not yet functioning. We have examined serum samples from kids at weekly intervals; a

progressive increase in enzymic activity occurred in the first weeks after birth.

We are grateful to Professor P. Hey, of the Royal College of Medicine, Baghdad, for the camel serum and to Mr I. H. Pattison, M.R.C.V.S., of the Agricultural Research Council Field Station, Compton, for the goat sera used in this study.

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Metabolism of 2α-Methylcortisone and 2α-Methylcortisol. By I. E. Bush and V. B. Mahesh.
(*The Radcliffe Infirmary, Oxford*)

2α-Methylation of corticosteroids and analogues possessing an 11β-hydroxyl group greatly enhances their biological activity (sodium retention, glycogen deposition and anti-inflammatory) while this step results in the loss of biological activity of steroids possessing an 11-keto group (Liddle, Richard & Tomkins, 1956; Dulin, Bowman & Stafford, 1957). In an earlier communication it was proposed that only 11β-hydroxy forms of the adrenocortical hormones are active at their target sites, and the low biological activity of 2α-methylcortisone was attributed to the absence of significant reduction of its 11-keto group to 11β-hydroxyl group, *in vivo* in man, as compared with cortisone (Bush & Mahesh, 1957). A similar observation was made on incubation of 2α-methylcortisone with rat liver microsome-supernatant fractions (Glenn, Stafford, Lyster & Bowman, 1957).

We have now examined, in detail, the urinary metabolites of 2α-methylcortisone and 2α-methylcortisol. 50 mg. each of these steroids were administered to male volunteers by mouth. Twenty-four-hour urine samples were extracted by the method of Bush & Gale (1957), and the extract hydrolysed by incubating with succus entericus of *Helix pomatia*. The final extract was fractionated and the components characterized by the use of paper chromatography. The 2α-methyl steroids characterized in urine after the administration of 2α-methylcortisone were: 2α-methylcortisone (I), 2α-methyl-3α:17α:20?:21-tetrahydroxy-5α-pregnan-11-one (II), 2α-methyl-17α:20?:21-trihydroxy-Δ^4-pregnen-3:11-dione (III) and its 6β-hydroxy derivative (IV), and 2α-methyl-Δ^4-androsten-3:11:17-trione (V), while 2α-methylcortisol (VI), 2α-methyl-11β:17α:20?:21-tetrahydroxy-Δ^4-pregnen-3-one (VII) and its 6β-hydroxy derivative (VIII), and 2α-methyl-11β-hydroxy-Δ^4-androsten-3:17-dione (IX) were obtained in quantities which were roughly 10% of their 11-keto analogues. Similarly, after administration of 2α-methylcortisol, the principle metabolites characterized were compounds (VI), (VII), (VIII) and (IX), while compounds (I), (III), (IV) and (V) were present in minor quantities.

The above results further confirm the hypothesis that the steric properties of ring A, and substituents therein, rather than the presence or absence of a Δ^4-3-ketone group, determine the specificity of biological oxidation-reduction of steroid 11-oxygen functions.

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Metabolism of 9α-Fluorocortisone and 9α-Fluorocortisol. By I. E. Bush and V. B. Mahesh.
(*The Radcliffe Infirmary, Oxford*)

Fried & Sabo (1954) and Goldfien, Laidlaw, Haydar, Renold & Thorn (1955) have demonstrated that the order of activities, in bioassays for glucocorticoids and in man, of 9α-halogenated cortisol is F > Cl > Br > H ≥ I. This striking correlation with the electronegativity series, with the exception of the large iodine atom which is often abnormal in such series of compounds, suggests that in the biological activity of the 11-oxygenated adrenocortical steroids, electronic shifts at (or near) the 11-oxygen function are important factors in determining the required physico-chemical properties. If, however, oxidation-reduction at C-11 is involved in the mechanism of action of these hormones, an alternative explanation of the action of 9α-halogen atoms is possible. If an enzyme is

responsible for this, the α-side would be the expected direction of attack, and the halogen atom might act by association with the active site on the enzyme by polar interaction or hydrogen bonding. This type of action would be very sensitive to steric factors, which would explain the negative effects of the very large iodine atom (Bush, 1956). Therefore a study of the metabolism of 9α-halogenated cortisone derivatives is of considerable interest.

Wienmann, Baulieu, Deltour & Jayle (1956) reported the isolation from urine of patients treated with 9α-fluorocortisol acetate of 9α-fluorocortisol and another compound (identity uncertain). We have now studied the metabolism of 9α-fluorocortisone and 9α-fluorocortisol, *in vivo* in man. 25 mg. of the steroids were administered to male volunteers and urine and plasma samples analysed using paper chromatography. On administration of 9α-fluorocortisone, the urinary metabolites characterized were: 9α-fluoro-11β:17α:20?:21-tetrahydroxy-Δ^4-pregnen-3-one, 9α-fluoro-3α:11β:17α:21-tetrahydroxy-5β-pregnan-20-one (9α-fluorotetrahydro-cortisol), 9α-fluoro-3α:11β:17α:21-tetrahydroxy-5α-pregnan-20-one (9α-fluoro-*allo*tetrahydro-cortisol), 9α-fluoro-3α:11β-dihydroxy-5β-androstan-17-one (9α-fluoro-11β-hydroxy-aetiocholanolone), 9α-fluoro:3α:11β-dihydroxy-5α-androstan-17-one (9α-fluoro-11β-hydroxy androsterone) and 9α-fluoro-3α-hydroxy-5β-androstan-11:17-dione (9α-fluoro-11-keto aetiocholanolone). 9α-Fluorocortisol gave all the above metabolites with the exception of the last named. In plasma only 9α-fluorocortisol was present in both cases.

The total absence of 9α-fluorocortisone and 9α-fluorotetrahydro-cortisone in urine as compared with the occurrence of the corresponding analogues after cortisone administration strongly suggests that oxidation-reduction equilibrium at C-11 of these compounds *in vivo* is in favour of the 11β-hydroxyl form. This is considered to be one of the major factors responsible for their biological activity. Also of significance is the much larger ratio of 5α:5β reduction of ring A, as compared to cortisone.

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The Coproporphyrin Precursor of Human Urine, and another Pigment formed from a Chromogen. By F. K. Herbert. (*Department of Pathology, King's College, Newcastle upon Tyne*)

In preparing extracts of the coproporphyrin precursor described by Saillet (1896), Raine (1950), Watson, de Mello, Schwartz, Hawkinson & Bossenmaier (1951) and Eriksen (1951*a*, *b*), the preformed coproporphyrin may be removed from ether extracts with aqueous HCl (0·25 to 1%, w/v), but some metalloporphyrins are not thus removed (Gray, 1951). The Zn complex is dissociated by higher concentrations of HCl, which also convert the precursor to porphyrin.

To distinguish between metalloporphyrin and precursor it is desirable to follow porphyrin formation spectroscopically. Since the precursor is readily converted to porphyrin, and light-sensitive, extracts have been made rapidly in a darkroom from fresh urine (2–4 hr. output). Concentrated extracts have been examined with a direct-vision spectroscope, using a simple attachment in which 3 ml. solution is viewed in depths up to 200 mm., which gives adequate sensitivity for normal urine.

A series of samples from twenty-three subjects (three normal, eight with lead poisoning, 12 miscellaneous) has been examined. Extracts in ether (with or without acetic acid), after removal of porphyrin with 1% HCl, were washed, concentrated, and examined at once for absorption spectrum. The precursor was then converted to porphyrin by a few minutes' exposure to ultraviolet light, and the spectrum examined again. Spectrum intensities were assessed from the least depth at which they were visible, and the relative intensities for coproporphyrin and its metal complexes taken into account. In all but five cases the initial examination showed no absorption bands, and irradiation produced coproporphyrin. The other five (all lead poisoning) showed metalloporphyrin initially; there was evidence that the precursor was also present, and in some instances metalloporphyrin formation was subsequent to conversion of the precursor during handling. Several properties of the precursor have been confirmed.

In addition, another pigment was formed from a chromogen under similar conditions in thirteen cases. Its spectrum in ether has an absorption band at 637 mμ (α-band of coproporphyrin 621 mμ read simultaneously). In aqueous HCl it has a band at 615 mμ. It has been separated from coproporphyrin but not isolated. It may be an analogue of the green porphyrins mentioned in the accompanying paper.

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Porphyrins in Human Cutaneous Porphyria and Bovine Porphyria. By F. K. HERBERT. (*Department of Pathology, King's College, Newcastle upon Tyne*)

The human patient is a man aged 30, with hydroea aestivale since age 24, and no hepatic crises. He was examined in January and October 1957; symptoms were slight throughout that year. The blood serum contained porphyrin, recognizable by its fluorescence emission spectrum. Total urinary porphyrin ranged from 8300 to 2700 μg./day, mainly octacarboxylic; porphobilinogen was absent. Fresh urine showed the absorption spectrum of neutral porphyrin and a second pigment (α band of porphyrin, 620 mμ, additional band, 640 mμ). In addition, minor porphyrin constituents and most of the other pigment occurred as chromogens. Faecal porphyrin amounted to 97–151 μg./g. dry weight, with octacarboxylic and tetracarboxylic porphyrins predominant, and dicarboxylics normal.

Urinary and faecal porphyrins were fractionated by repeated column chromatography of their methyl esters on MgO and $CaCO_3$. They fell into four groups: porphyrins with spectroscopic and chromatographic properties in regular series from octacarboxylics to dicarboxylics (P); metalloporphyrins (traces); green porphyrins; and other porphyrin esters (R).

The major component from urine was a mixture of uroporphyrin III and 'pseudouroporphyrin', from which the uroporphyrin was separated by fractional crystallization. Minor P fractions also occurred, including coproporphyrin III. A green porphyrin, isolated in small amount, explained the composite absorption spectrum of the urine.

The major components from faeces were 'pseudouroporphyrin' and coproporphyrin III. Minor P fractions occurred, including a dicarboxylic. A green porphyrin was isolated in small amount.

The 'pseudouroporphyrins' of this case resemble porphyrins found by Canivet & Rimington (1953) and Benson, Foster & Prunty (1954) in porphyria cutanea tarda, and the 'pseudouroporphyrin' of Falk, Dresel, Benson & Knight (1956).

The bovine material was bone, from a carcass of unknown source, with deposits typical of porphyria. Porphyrin from this was fractionated (as methyl ester). The main component was uroporphyrin (I and III). Traces of other porphyrins were present, and a green porphyrin was isolated in small amount.

The green porphyrins obtained from the bovine bone and the patient's urine appear identical, and that from the patient's faeces closely similar. In general type they resemble the hepatic green porphyrins of Schwartz & Ikeda (1955).

Thanks are due to Dr H. A. Dewar for opportunity to examine the patient, to Mr P. Trinder for the bone, and to Professor C. Rimington and Professor C. H. Gray for standard porphyrin samples.

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The Lipolytic Activity of Rat Plasma after the Intravenous Injection of various Dextran Sulphates. By D. S. ROBINSON, P. M. HARRIS and C. R. RICKETTS. (*Sir William Dunn School of Pathology, Oxford and Birmingham Accident Hospital, Birmingham*)

The clearing of chyle *in vitro* by plasma from an animal which has received an intravenous injection of heparin is due to a lipolytic process and is accompanied by the hydrolysis of the triglyceride of the chylomicra and by the production of unesterified fatty acids. Clearing occurs because the unesterified fatty acids which are released go into solution as a complex with the plasma albumin (Robinson & French, 1957).

Plasma from an animal into which dextran sulphate is injected also clears chyle by a similar lipolytic process. In the present communication some studies on the lipolytic activity produced by dextran sulphates of different molecular weights and sulphur contents are reported.

Three preparations of dextran sulphate of low molecular weight and containing 16·2, 13·9 and 2·2% of sulphur respectively were injected into

rats. The clearing activity of the plasma from these animals decreased as the sulphur content of the injected dextran sulphate fell. Thus, the fraction containing 16·2% sulphur had 60–70% of the activity of heparin and that containing 2·2% sulphur had about 10% of the activity of heparin. The dextran sulphate preparations containing 16·2 and 13·9% of sulphur had anticoagulant activity, whereas the preparations containing 2·2% of sulphur had no anticoagulant activity.

Several dextran sulphates of the same sulphur content but different molecular weights, ranging from about 10 000 to several million (Ricketts, 1952), were injected into rats. The clearing and lipolytic activity *in vitro* of the plasma from these animals fell as the molecular weight of the dextran sulphate preparation rose. Evidence will be presented which suggests that both the high and low molecular weight preparations are equally effective in producing clearing and lipolytic activity in the plasma, but that the high molecular weight dextran sulphates inhibit the lipase which they induce (cf. Walton, 1952), so that the clearing activity detectable *in vitro* is low. The inhibitory effect is abolished in the presence of an adequate amount of protamine sulphate.

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The Effect of Opsopyrroledicarboxylic Acid (OPD) on the Synthesis of Haem. By J. J. Scott and Audrey T. Smith. (*Department of Chemical Pathology, St Mary's Hospital Medical School, London, W. 2*)

OPD (MacDonald & MacDonald, 1955) has the same β-substituents as porphobilinogen but both α-positions are unsubstituted. Jackson & MacDonald (1957) suggested that OPD might be involved coenzymically in the biosynthesis of series III porphyrins. The mechanism proposed requires a directed condensation of OPD on the aminomethyl carbon atom of a linear tetrapyrromethane formed from porphobilinogen, removal of an OPD residue from the other end of the resulting pentapyrromethane, and ring closure. Porphyrins formed in this way from ^{14}C-succinate or ^{14}C-glycine would still exhibit the characteristic patterns of labelling (summarized by Shemin, 1955) if OPD were formed from porphobilinogen. However, in the presence of an excess of unlabelled OPD, while the methene-bridge labelling would be unaffected, labelling in one of the four pyrrole rings should disappear. This would cause a decrease in the radioactivity of the porphyrin and, when formed from [α-^{14}C]glycine, an increase in the ratio (total bridge-^{14}C)/(total porphyrin-^{14}C), the value of which can be obtained by degrading the porphyrin and is normally 0·5.

OPD was one of several related pyrroles tested by Bogorad (1957) in a plant-enzyme system containing a porphobilinogen deaminase (which forms 'uroporphyrinogen' I) and other enzymes; it was found to be a competitive inhibitor of the deaminase. We have incubated haemolysates prepared from anaemic fowls with OPD, glycine and succinate, isolating haem after incubation, by conversion to protoporphyrin methyl ester copper complex. The following findings, which we consider completely exclude OPD as an intermediate, will be presented: Although the radioactivity of the haem falls with increasing OPD concentration, the decrease is the same whether labelled glycine or succinate is used, indicating inhibition, but no dilution, by OPD. After incubation with [α-^{14}C]-glycine, the ratio (total bridge-^{14}C)/(total porphyrin-^{14}C) is independent of OPD concentration and close to 0·5. Finally, ^{14}C-OPD is not incorporated into haem.

OPD does not appear to penetrate either into red cells, as judged by the absence of inhibition of haem synthesis, or into several gram positive and negative micro-organisms, as judged by failure to affect their growth (we are grateful to Dr Brumfitt for these latter tests). It has not been excluded that OPD is formed in red cell lysates during haem synthesis.

We thank Dr S. F. MacDonald for gifts of compounds.

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Some Observations on the $2\frac{2}{3}$-Calcium Orthophosphate (Octocalcium Phosphate). By S. L. ROWLES. (*M.R.C. External Staff, Chemistry Laboratory, Royal Veterinary and Agricultural College, Copenhagen, Denmark, and Department of Dental Pathology, University of Birmingham*)

The existence of a calcium orthophosphate with composition $Ca_4H(PO_4)_3$, aq., for long disputed, has been conclusively demonstrated by Bjerrum (1936, 1958). Recently Jensen & Hansen (1957) detected its presence in Copenhagen dental calculus by careful examination of Debye-Scherrer powder diagrams.

Examination of eighteen samples of dental calculus from the London area using the Guinier monochromatic X-ray diffraction technique, which is capable of detecting less than 5% $2\frac{2}{3}$-phosphate in mixtures, showed the presence of this compound in every sample, while in four it was the major crystalline constituent.

By the action of water upon the secondary calcium phosphate, brushite, crystals of $2\frac{2}{3}$-phosphate are formed on the surface of the brushite, but their appearance is inhibited or delayed in the presence of Cu^{2+} at 10^{-5} M, or Mg^{2+} at 10^{-3} M. The solubility products (Bjerrum) calculated from analyses on solutions from which $2\frac{2}{3}$-phosphate had been precipitated were found to agree more closely with those found under dissolution conditions by Bjerrum (1936). Some of the dissolution values were anomalous and suggested that under these conditions the solution was not in equilibrium with the $2\frac{2}{3}$-phosphate phase.

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DEMONSTRATIONS

Improved Methods for the Paper Chromatography of Organic Acids. By G. J. LAWSON and R. D. HARTLEY. (*Department of Mining, Birmingham University*)

The Concentration of Protein Solutions by Ultrafiltration through Dialysis Tubing. By G. H. GRANT (*Department of Pathology, Royal Salop Infirmary, Shrewsbury*), D. S. ROWE and D. R. STANWORTH. (*Department of Experimental Pathology, Medical School, Birmingham*)

It is frequently necessary to concentrate dilute protein solutions, such as those obtained from column chromatographic or electrophoretic fractionations, prior to further experiments. We have modified the apparatus previously described by one of us (Grant, 1957) to permit the simultaneous concentration and dialysis of several solutions. Various protein solutions, including diluted human serum, concentrated in this way, showed minimal alteration in their compositions as revealed by ultracentrifugal, electrophoretic and immunological analyses.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **Annual General Meeting** *of the Biochemical Society was held in the Department of Biochemistry, University College, Gower Street, London, W.C.* 1, *on Friday,* 18 *April* 1958, *starting at* 10.45 *a.m.; the Meeting continued on Saturday,* 19 *April* 1958, *starting at* 10.30 *a.m. The following papers were read:*

COMMUNICATIONS

Absorption of Cystine and Cysteine from Rat Small Intestine. By M. W. Neil. (*Department of Biochemistry, London Hospital Medical College, Turner Street, London, E.* 1)

Studies have been made in this laboratory on the absorption of cystine and cysteine from the small intestine of the rat using both an *in situ* lumen perfusion technique (Neil, 1957) and the everted sac technique of Wilson & Wiseman (1954).

Using the *in situ* preparation the rates of absorption for both amino acids were found to increase approximately linearly with increasing initial concentration in the perfusion fluid. The upper concentration limit for cystine was fixed by its solubility in physiological saline at pH 7·4. Supersaturated solutions which were stable during the experimental period could not be prepared. When the perfusion fluid contained cysteine it was equilibrated with 95 % N_2, 5 % CO_2 and not with 95 % O_2, 5 % CO_2. The rates of absorption from different regions of the rat small intestine varied for both amino acids in the order: duodenum > jejunum > ileum. The D-isomers were absorbed from the duodenum at significantly lower rates than the L-isomers. 2:4-Dinitrophenol (DNP) added to the perfusion fluid (3×10^{-4}M) after a control period had no effect on the rates of absorption of the L-isomers. It is interesting to note that DNP also had no effect on the rate of absorption of D-glucose from rat jejunum in this preparation, whereas phlorrhizin (10^{-3}M) caused practically complete inhibition. The absorption of L-methionine was similarly unaffected by the presence of DNP. Controls on the preparation were carried out by routine determinations of tissue potassium and water and by histological examination. The intracellular potassium concentration (about 430 m-equiv./kg. fat-free dry tissue) was well maintained, but the water content rose from about 78·5 to 81·8 % (calculated on a fat-free basis) during 2–4 hr. perfusion periods.

Using the everted sac technique, control experiments showed a decrease in intracellular potassium and an increase in the water content during 1 hr. incubation. Further experiments demonstrated that these changes occurred in the first 10 min. of incubation, and that a new steady state was rapidly established in which the potassium and water contents of the tissue were about 350 m-equiv./kg. and 84 % respectively. The serosal/mucosal amino acid concentration ratios for jejunal sacs rose during 1 hr. incubation from 1·0 to 1·1 (L-methionine) and 2·9 (L-cystine) but fell to 0·89 for D-cystine. DNP (3×10^{-4}M) caused catastrophic damage to the sacs, the potassium contents of both the whole tissue and the mucosal cells alone rapidly fell to about 50 % of normal. The effects of DNP on absorption processes in such preparations are probably unspecific in view of the gross generalized damage caused.

It seems probable from these studies that both L-cystine and L-cysteine are actively absorbed from the rat small intestine, possibly by a common mechanism.

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Partial Acid Hydrolysis of the Cell-wall of *Micrococcus lysodeikticus*. By H. R. Perkins and H. J. Rogers. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Micrococcus lysodeikticus (strain NCTC 2665) was grown by incubation overnight at 30° of nutrient agar surface cultures, harvested, and washed twice with water. The cell-walls were then prepared by the method of Cummins & Harris (1956). The final product was dried at 105° overnight. By acid hydrolysis and chromatographic examination it was found to contain alanine (20 %), glutamic acid (13 %), glycine (11 %), lysine (20 %), glucosamine (10 %), muramic acid (8 %) and glucose (4·5 %).

The amino acids were estimated by the elution of ninhydrin-stained spots in a modification of the method of Kay, Harris & Entenman (1956) and glucosamine and muramic acid by the method of

J. T. Park (personal communication). Glucose was estimated as reducing sugar, after passing hydrolysates through columns of Dowex 50 (H^+) to remove amino acids and amino-sugars. Liberation of the various components was followed during hydrolysis by 2N-H_2SO_4 at 100°. It was found that the amount of material measurable as *N*-acetylhexosamine reached a maximum value equivalent to 14% of the cell-wall after 20 min. hydrolysis, and then declined with liberation of free hexosamine. Glucose was set free very rapidly, 90% within 20 min. and 100% in 60 min. Examination of cell-wall hydrolysed for 20 min. showed that 98% of the material giving an *N*-acetylhexosamine colour was dialysable. Most of this material could be eluted from cation exchange resins by water and was shown by paper chromatography to be *N*-acetylglucosamine and *N*-acetylmuramic acid. The remainder appeared in the first fractions eluted by 0·3N-HCl (i.e. before the amino-sugars). These fractions showed several slow-running spots on paper chromatography in butanol–acetic acid–water, which were visualized either with ninhydrin or with the Elson–Morgan reagents.

Each of the spots was eluted from the paper and examined by paper electrophoresis. Several fractions were obtained as follows: Fraction A, which gave a weak reaction for hexosamine before hydrolysis, contained principally glucosamine and muramic acid in equimolecular proportions. Fraction B, which also gave a weak hexosamine reaction before hydrolysis, contained glucosamine, muramic acid and glycine in the molecular proportions of 2:1:2. Fraction C, which gave a *N*-acetylhexosamine reaction before hydrolysis, contained alanine, glutamic acid, glycine, lysine and muramic acid in the proportions 4:2:3:2:2.

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Tracer Studies of the Metabolism of the Tumour Inhibitor Triethylenethiophosphoramide.

By A. W. Craig, B. W. Fox and H. Jackson. (*E.R.T. Department, Christie Hospital and Holt Radium Institute, Withington, Manchester* 20)

In an earlier publication a study of the metabolism of triethylenephosphoramide labelled with radioactive phosphorus was reported (Craig & Jackson, 1955). In the rat over 80% of the radioactivity is excreted in the urine in 24 hr., mainly as unchanged drug. This suggested that although triethylenephosphoramide is highly effective as an inhibitor of certain experimental tumours in the rat, it is not particularly reactive *in vivo*.

Tracer studies in mice with another ethyleneimino compound, triethylenemelamine, have been made by Nadkarni, Goldenthal & Smith (1954). Only cyanuric acid was found in the urine, from which they concluded that compounds of this type have a marked chemical reactivity in the body. More recently Heidelberger and his associates examined the metabolism in tumour patients of labelled morpholinodiethylenethiophosphoramide. They identified substantial amounts of morpholinodiethylenephosphoramide in the urine, the sulphur in the drug having been replaced by oxygen; the major metabolite in the rat was the same (Maller & Heidelberger, 1957).

In view of the above differences we have now examined the metabolism in three species—dog, rat and mouse—of triethylenethiophosphoramide labelled with radioactive phosphorus. Radioactive material was excreted very rapidly in all cases, but whereas the main urinary metabolite in the rat appears to be triethylenephosphoramide, i.e. the sulphur of the drug having been replaced by oxygen, in the mouse the drug is completely broken down to inorganic phosphate. Urine from the dog contained not only triethylenephosphoramide but also a considerable proportion of an unidentified metabolite. These differences in the metabolism of compounds containing ethyleneimino-groups may help to explain the observed variations in their effectiveness against tumours in different species. Conclusions as to the chemical reactivity of these compounds *in vivo* can be misleading if based on their metabolic fate in a single species.

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Biochemical Differentiation between Radio-sensitive and Non-sensitive Tissues in the Rat.
By W. A. Creasey and L. A. Stocken. (*Department of Biochemistry, University of Oxford*)

In order to obtain a clue to the fundamental lesion in radiation damage, a comparison has been made between the enzymic composition of nuclei prepared from radio-sensitive and non-sensitive tissues. About thirty nuclear enzymes are known, but up to the present we have only one test system which differentiates between the two kinds of tissue.

Osawa, Allfrey & Mirsky (1957) have shown that calf-thymus nuclei, prepared in a sucrose medium, will phosphorylate the intranuclear adenine, guanine and uridine monophosphates to the corresponding triphosphates when shaken aerobically at 0° in the medium. We have prepared nuclei from the radio-sensitive tissues thymus, spleen, lymph node, intestinal mucosa and bone marrow and from the non-sensitive liver, kidney, brain and pancreas. The nuclear suspensions were shaken for 30–40 min. at 0° and samples obtained at the beginning and end of the experiment were freed from protein by means of perchloric acid. Labile phosphate was determined by heating the samples in N-HCl at 100° for 10 min. The rate of generation of labile phosphate expressed in μg. phosphorus/mg. deoxyribonucleic acid phosphorus/min. is for thymus 0·31, spleen 0·71, lymph node 1·83, bone marrow 8·3 and intestinal mucosa 0·31. The non-sensitive tissues do not show any increase.

The effect of small doses of radiation on this nuclear phenomenon is quite marked. 100 r. total body radiation given to rats which are killed 1 hr. later, completely suppresses the high-energy phosphate generation, while even as low a dose as 25 r. leads to a 50–80 % inhibition of the reaction.

As is to be expected, there is a recovery of the reaction in the tissues of rats exposed to sublethal doses but not in those given 1000 r. Such recovery has been followed in the spleen and thymus after 100 r. whole body irradiation. Recovery commences in the third day and is complete in about 100 hr. after the exposure to X-rays.

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The Purification of Pancreatic Lipase using Zone Electrophoresis on Plastic Sponge.
By E. D. Wills. (*Department of Biochemistry, The Medical College of St Bartholomew's Hospital, London, E.C.* 1)

Pancreatic lipase has been purified by means of a simple method based on one originally described by Glick & King (1933).

A standard acetone powder of hog pancreas prepared as described by Wills (1954) was extracted 4 times with distilled water. The pH of the combined extracts was adjusted to 5·0 and $MgSO_4$, $7H_2O$ (1 g./ml.) was added, causing the formation of a precipitate containing practically all the original lipase activity. This precipitate was filtered off, suspended in a small volume of water and dialysed against acetic acid–sodium acetate buffer (10^{-3} M, pH 5·5) for 24–48 hr. The suspension clarified initially, but subsequently a precipitate formed which was centrifuged and dissolved in a small volume of NaCl (5 %, w/v). The resulting solution had a lipase activity/mg. protein ratio about 20 times that of the original extract.

Further purification of the enzyme was achieved by electrophoresis on plastic sponge using an apparatus similar to that outlined by Davidson (1957). This technique will be described.

At pH 9·0 in either borate or tris-HCl buffer the purified preparation was separated into several components by this technique, but the major portion of the lipase activity was found to be associated with one component only, whose electrophoretic mobility suggests that its isoelectric pH is about 4·5–5·0. The purified component was found to hydrolyse a variety of triglycerides and monoglycerides and to exhibit generally a low specificity similar to that of the crude extract.

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The Oxidation of Tricarboxylic Acid Cycle Intermediates by *Pseudomonas aeruginosa*. By PAULINE MEADOW* and PATRICIA CLARKE. (*Department of Biochemistry, University College London*)

Pseudomonas aeruginosa was grown in the presence of various intermediates of the tricarboxylic acid cycle. Whole cells and extracts were then compared for their activity towards these substrates in the Warburg apparatus.

All the substrates tested (succinate, fumarate, malate, pyruvate, acetate, α-oxoglutarate and citrate) were oxidized linearly from the time of adding the substrate to citrate-grown cells. Cells grown with succinate, pyruvate, fumarate, malate and α-oxoglutarate oxidized citrate only after a lag period of about 3 hr. Addition of 1 μmol. of any readily oxidized substrate shortened the lag period to about 2 hr. Similar, though shorter (10 min.), lag periods preceded oxidation of acetate by fumarate-, pyruvate-, malate- and α-oxoglutarate-grown cells, and also the oxidation of succinate and fumarate by acetate-grown cells. Addition of chloramphenicol (40 μg./ml.) or 8-azaguanine, (100 μg./ml.) prevented these adaptations to the substrates. All other substrates were oxidized without a lag period.

Extracts of cells grown on the various substrates were prepared by disintegration in the bacterial press, the debris being removed by centrifugation. Cell-free extracts were thus obtained which oxidized citrate, fumarate, succinate, malate and acetate linearly from the time of substrate addition, even from those bacteria which showed apparent adaptation in whole cells. All the extracts, including those from pyruvate-grown cells, were inactive towards pyruvate. Chloramphenicol and 8-azaguanine had no effect on any of the extracts.

Kogut & Podoski (1953) and Barrett & Kallio (1953) concluded from their experiments with whole cells and extracts of *P. fluorescens* that the lag observed in the oxidation of certain tricarboxylic acid cycle intermediates by whole cells involved making the substrates accessible to the enzyme, either across the cell membrane or to some special site within the cell. More recent work (Cohen & Monod, 1957) has shown that in *Escherichia coli* there are proteins (permeases) which are concerned solely with the transport of stereospecific substrates across the cell-wall. The effects of 8-azaguanine and chloramphenicol in preventing the adaptation of *P. aeruginosa* to certain substrates support the hypothesis that during these lag periods the necessary permeases are being synthesised.

* Beit Memorial Fellow.

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Determination of Liver Glycogen by the Sulphuric Acid Reaction. By C. E. SEARLE and D. L. WOODHOUSE. (*Cancer Research Laboratories, The Medical School, Birmingham*, 15)

Kemp & Kits van Heijningen (1954) described an attractive micro-method for determining liver glycogen in which a deproteinized homogenate is heated with 3 vol. of 96% H_2SO_4 giving a bluish pink colour, the optical density of which is measured at 520 mμ. However, when using this method to investigate the distribution of glycogen in the livers of rats fed 4-dimethylaminoazobenzene, orange-pink solutions were obtained, although glucose gave the correct colour. In order to investigate whether this alteration might indicate qualitative changes in liver carbohydrates during carcinogenesis, experiments on the applicability of the method have been carried out.

Solutions obtained from tumorous liver samples showed high absorption over much of the 400–500 mμ range, the 520 mμ maximum being sometimes lower than a maximum at 480 mμ. Solutions from normal rat livers also had somewhat higher relative absorption between 400 and 500 mμ than those from standard glucose solutions. The differences were more marked in the absorption spectra of solutions from muscle (Kemp & Kits van Heijningen, 1954) or blood (Mendel, Kemp & Myers, 1954), though visually the colours were normal.

The method is stated (Mendel *et al.* 1954) to be specific for certain hexoses and their derivatives, but since arabinose, xylose and rhamnose give pale yellow colours it seemed likely that ribose and deoxyribose would also react. Solutions of RNA and DNA gave pale yellow colours after treating by the deproteinization and colorimetric procedures, this being ascribed to the pentose units, since pure purines and pyrimidines gave no colour whatever. DNA gave a peak at 472 mμ, and RNA a number of smaller ill-defined maxima in the 400–600 mμ range. Both showed appreciable absorption at

520 mμ, equivalent to about 20 % of that given by an equal concentration of glucose.

While the method evidently yields satisfactory values in suitable tissues, errors due to the above and other factors can be great, particularly when the glycogen content is low.

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The Determination of 11-Deoxy-17-oxosteroids in Urine from Adrenalectomized Women. By R. D. Bulbrook, F. C. Greenwood and B. S. Thomas. (*Imperial Cancer Research Fund, Royal College of Surgeons, Lincoln's Inn Fields, London, W.C.* 2)

The method of Kellie & Wade (1957) was tested to determine whether it could be used for the accurate estimation of dehydro*epi*androsterone, androsterone and aetiocholanolone in urine from adrenalectomized women. In our hands the method was found suitable only for qualitative detection of these compounds in this type of urine.

In most cases considerable amounts of pigment were present in the urinary extracts. Removal of the pigment by Girard separation occasionally led to acetate formation, even in the cold. The acetates of the 11-oxygenated 17-oxosteroids were eluted with the 11-deoxy steroids and the Girard separation was therefore abandoned. Since most of the interference was due to indigo red, this pigment was removed by reduction with alkaline sodium dithionite. A more serious source of interference was traced to unidentified Zimmerman chromogens, eluted from the column with the 11-deoxy-17-oxosteroids. Ten such chromogens have been found, despite either Girard separation or dithionite reduction.

When eluates from the column were further resolved by paper chromatography it was found that most patients continued to excrete aetiocholanolone after adrenalectomy. Androsterone was rarely found. Dehydro*epi*androsterone was found in one case where there was other evidence for incomplete adrenalectomy.

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Oxygen Consumption and Loss of Activity of Heart-muscle Succinic Oxidase Preparations Deficient in Inorganic Phosphate. By M. B. Thorn. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

Heart-muscle preparations containing little inorganic phosphate readily lose succinic oxidase and succinate-methylene blue reductase activity on storage (Ball & Cooper, 1949; Thorn, 1956). Succinic oxidase activity in low-phosphate media is activated by compounds (e.g. phosphate, histidine, ethylenediaminetetraacetate) which neither affect succinate-methylene blue reductase activity (Keilin & Hartree, 1949; Slater, 1949*a*; Bonner, 1954) nor restore activity to aged preparations. The problems of ageing and of activation, although perhaps related, are therefore different.

Ball & Cooper (1949) found that phosphate and fluoride together were particularly effective in protecting their low-phosphate preparations from loss of activity. Slater & Bonner (1952) showed that phosphate and fluoride together competitively inhibited succinic dehydrogenase, and this observation, coupled with the fact that competitive inhibitors of succinic dehydrogenase protect it from sulphydryl reagents (Hopkins, Morgan & Lutwak-Mann, 1938; Potter & DuBois, 1943), prompted the hypothesis, here advanced, that low-phosphate preparations might lose activity because of oxidation of sulphydryl groups. This hypothesis was strengthened by the following observations: (*a*) phosphate and fluoride together protected succinic dehydrogenase from inhibition by *p*-aminophenylarsenoxide (this is supported by the detailed work of Stoppani & Brignone (1956)), (*b*) malonate was a highly effective protective agent, (*c*) the enzyme was well protected under anaerobic conditions. On the other hand, the conclusion of Ball & Cooper (1949), that loss of activity was the result of breakdown of a

phosphorylated compound, was weakened, in the present work, by the finding that competitive inhibitors and anaerobic conditions did not significantly affect the appearance of inorganic phosphate in the preparation. The 'oxidation hypothesis' is not in conflict with many studies (e.g. MacFarlane, 1950; Nygaard, 1953; Edwards & Ball, 1954) which have shown, by the use of specific phospholipases, that phospholipids are important structural elements of these enzyme-bearing particles.

The oxygen consumption of heart-muscle preparations in the absence of added substrate (Q_{O_2} at $37° < 1$) has characteristics of a non-enzymic autoxidation, and is believed to be closely related to the loss of activity on ageing. The effects of this oxygen consumption will be compared with the effects (Slater, 1949*b*) of oxidizing agents (*o*-iodosobenzoate and oxidized glutathione), and of low concentrations of BAL in air (Slater, 1949*c*).

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Ascorbic Oxidase Activity in Extracts from Higher Plants. By V. S. Butt and Mary Hallaway. (*Department of Botany, University of Oxford*)

Ascorbate is readily oxidized in tissue extracts from higher plants. Agents which may catalyse the reaction include not only ascorbic oxidase, but also cytochrome oxidase, peroxidase, polyphenolase, unbound copper ions and copper-protein complexes. As the first step in an investigation of the function of ascorbic oxidase in plants, methods have been developed to distinguish the activity of this enzyme from that of the other catalysts.

From a comparative study of the kinetics of copper-ion catalysis and of that by an enzyme extracted and purified from vegetable marrow, it is proposed that ascorbic oxidase activity can be recognized in homogenates buffered at pH 6·0 by (i) a rapid copper turnover, (ii) identical rates of oxidation of 4 mM- and 40 mM-ascorbate, (iii) its insensitivity to 2 mM-EDTA, (iv) its complete inhibition by 0·1 mM-sodium diethyldithiocarbamate and by 1 mM-8-hydroxyquinoline, (v) its inability to catalyse the oxidation of 4 mM-catechol or 4 mM-quinol.

These tests have been applied to a selection of plant extracts, in most of which the enzyme was found to be active. Deviations from these characteristics have been observed in preparations from potato, cabbage leaf and barley seedlings. In squash juice, the reaction inactivation reported for the purified enzyme (Powers & Dawson, 1943) does not take place; in all crude extracts examined, the oxidation proceeds to completion.

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The Absorption of Vitamin B_{12} in the Rat. By M. E. Coates and E. S. Holdsworth. (*National Institute for Research in Dairying, Shinfield, nr Reading, Berkshire*)

Adult rats and ^{60}Co-labelled vitamin B_{12} were used in these experiments and all ^{60}Co determinations were made by scintillation counting. The radioactivity found in the gut wall after oral dosing with 25 μmg. vitamin B_{12} confirmed the finding of Booth, Chanarin, Anderson & Mollin (1957) among others that the ileum is the most active site of absorption of the vitamin in the rat. The total counts in lymph obtained by cannulation of the main mesenteric lymphatic duct showed that lymph is much less important than the blood stream as a route of absorption.

In an extension of our previous work (Holdsworth & Coates, 1956), three intestinal loops of equal length, each containing 10 μmg. vitamin B_{12}, were established in rats. The animals were killed after 18 hr. and the loops and various organs examined. The greatest absorption took place in

the middle and lower loops. Rats which received vitamin B_{12} alone absorbed about 13% of the dose during the experiment. Addition of rat-stomach extract increased absorption to 40%, but clinically active preparations of pig intrinsic factor reduced absorption to 9%. With a sample of human gastric juice known to be active in pernicious anaemia the absorption in the two upper loops was not greatly different whether the vitamin B_{12} was given alone or with human gastric juice. In the lowest loop, however, human gastric juice increased absorption from 10 to 24%. This observation might provide the basis for any assay of intrinsic factor activity of human gastric juice.

The maximal absorption of vitamin B_{12} within a defined area suggested the existence of special receptor cells in that region. Preparations of washed surviving mucosal cells from the three portions of the small intestine were incubated for 1 hr. with vitamin B_{12} at 37° in Krebs–Ringer bicarbonate glucose. The cells were separated and washed. Results followed closely those with live animals, in that the greatest uptake of vitamin B_{12} occurred in cells from the middle portion of the intestine; uptake was nearly doubled by rat-stomach extract and partially inhibited by pig intrinsic factor. Contrary to the *in vivo* findings, human gastric juice inhibited uptake of vitamin B_{12} by the mucosal cells, particularly in the lowest portion of the gut where it had been most active in the live animal.

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Metabolism of further 11-Oxygenated Steroids. By I. E. Bush and V. B. Mahesh. (*Radcliffe Infirmary, Oxford*)

Reduction of the steroid 11-ketone group *in vivo* is of interest because it seems likely that 11-oxo adrenal steroids must be reduced to the 11β-ols before biological activity appears (Bush, 1956; Bush & Mahesh, 1957; Glenn, Stafford, Lyster & Bowman, 1957). It has been suggested that this reaction *in vivo* occurs only with steroids having a relatively flat α-surface over rings A and B (Bush, 1956; Bush & Mahesh, 1957). Pursuing this topic we have now examined the urinary metabolites of 2α-methyladrenosterone (I), *allo*tetrahydrocortisone (II), tetrahydrocortisone (III), and prednisone (IV) after oral administration to young men.

40 mg. I gave rise to 2α-methyl-11β-hydroxyandrostenedione, I, 2α-methyl-17?-hydroxyandrost-4-ene-3:11-dione, 2α-methyl-11β:17?-dihydroxyandrost-4-en-3-one, 2α-methyl-3α-hydroxy-5α-androstane-11:17-dione, and an unidentified diol (total 12 mg.). The ratio I:2α-methyl-11β-hydroxyandrostenedione was 1·45. Reduction of the II-ketone was thus more complete than in the case of 2α-methylcortisone (Bush & Mahesh, 1957).

50 mg. II gave 1·1 mg. II, 9·2 mg. *allo*tetrahydrocortisol, and 0·97 mg. 11β-hydroxyandrosterone. 60 mg. III gave 10·7 mg. III, 0·6 mg. tetrahydrocortisol, and 0·87 mg. 11-oxoaetiocholanolone. 50 mg. IV gave, among others, 1·0 mg. IV and 1·2 mg. prednisolone.

In these experiments the 11-ketone:11β-ol ratio was found in each case to be the same in plasma as in the urine.

These results confirm the hypothesis that the main factor determining the possibility of reduction of steroid 11-ketone groups in man is the stereochemistry of the AB ring system (Bush, 1956) rather than the presence or absence of a Δ^4-3-ketone group (Fukushima, Hubener & Gallagher, 1956). Prednisone is particularly interesting in that the angulation of the A/B ring junction is intermediate between the flat *trans* and the fully bent *cis* forms of the saturated 3-hydroxy steroids.

The relative incompleteness of reduction of prednisone (50%, against 90% of cortisone) was correlated with a significant reduction in the biological activity of prednisone (11-oxo) (judged by adrenal suppression) compared with prednisolone (11β-ol). This is similar to, but less striking than, the same associated findings with 2α-methylcortisone (Bush & Mahesh, 1957, 1958).

These results also confirm the supposition (Dorfman, 1954) that 17-oxosteroids can arise from saturated 3-hydroxy-steroids like II and III.

With the doses employed it is reasonable to assume that the observed 11-oxo:11β-hydroxyl ratios are determined, with these steroids, by the rate of 11-oxo reduction and not to any significant extent by differences in redox potential of the 11-oxygen functions of the steroids concerned.

We are grateful in this and earlier studies for gifts of steroids from the Upjohn Company, U.S.A., Dr B. Hems (Glaxo Laboratories), Dr R. K. Callow (M.R.C.), Dr C. Hewett (Organon) and Dr M. Elyan (Roussel Laboratories).

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Indolylacetamide—a Chromatographic Artifact from the Natural Indoles, Indolylacetylglucosiduronic Acid and Indolylpyruvic acid. By J. B. Jepson. (*Courtauld Institute, Middlesex Hospital Medical School, London, W.* 1)

During investigations into the biochemical lesion of Hartnup disease (Baron, Dent, Harris, Hart & Jepson, 1956; Jepson, 1956), normal subjects have been given single doses of indolylacetic acid (IAA) and the subsequent changes in urinary excretion patterns examined.

When such urines were subjected to two-way paper chromatography in *iso*propanol–ammonia/butanol–acetic acid, a solvent pair recommended for the separation of indoles (Jepson, 1958), 3-indolylacetamide was found on the chromatogram. This seemed an unlikely metabolite and could not be isolated from the urine itself. Further experiments showed that it only appeared when *ammoniacal* solvents were used for the chromatography. If these were avoided, a new indolic component was revealed, subsequently isolated from the urine by solvent partition and shown to be *indolylacetyl glucosiduronic acid*, a new human conjugate of IAA.

This acyl-glucuronide is extremely rapidly converted to glucuronic acid and IAA by alkali, to indolylacetamide by ammonia, and to indolylacethydroxamic acid by neutral hydroxylamine—a reaction used for its determination in urine (cf. Schachter, 1957). It is hydrolysed by β-glucuronidase preparations. A 500 mg. oral dose of IAA is largely accounted for in the urine of the subsequent 24 hr.—200 mg. as the glucuronide and the rest as free IAA and indolylacetylglutamine (Jepson, 1956). Many normal urines contain traces of the glucuronide, which changes to IAA and indolylacetamide if the urine becomes ammoniacal or on chromatography in ammonia. Urines from cases of Hartnup disease have only moderate amounts.

In a search for other natural indoles which would yield IAA or indolylacetamide on chromatography in ammoniacal solvents, the alkaline degradation of indolylpyruvic acid (IPyA) was reinvestigated [in collaboration with Dr Joyce Bentley, Marine Laboratory, Aberdeen]. Chromatography in *iso*propanol-ammonia of fresh IPyA gave indolylacetamide in 5–10% yield. As well as IAA and indolylglycollic acid (already reported by Bentley, Farrar, Housley, Smith & Taylor, 1956), another surprising product was indole-3-carboxylic acid. It seems unlikely that any IPyA was left (contrast the conclusions of Stowe & Thimann, 1954; Schwarz & Bitancourt, 1957) since no area of a one-way chromatogram gave indolyl-lactic acid after reduction with sodium borohydride—a device which succeeded with chromatograms from acid solvents.

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The Free Glycine Metabolic Pool in Man. By J. C. Crawhall and R. W. E. Watts. (*The Medical Unit, St Bartholomew's Hospital, London, E.C.* 1)

Study of the free glycine metabolic pool in man has been handicapped by the lack of a satisfactory method of sampling the pool after the administration of the isotopically labelled amino acid, The hippuric acid excreted after a loading dose of sodium benzoate has been used for this purpose (Gray & Neuberger, 1950; Berlin, Hewitt & Lotz, 1954). The validity of this can be questioned (see, for example, Gray & Neuberger, 1950), and we suggest that the uncombined urinary glycine may be regarded as a representative sample of the free glycine metabolic pool. In the present work, the

isotope content of the urinary hippuric acid after the oral administration of ^{15}N-labelled glycine has been compared with that of the uncombined glycine in the same urine specimen; loading doses of benzoate were not given. After extraction of the hippuric acid, the uncombined urinary amino-acids were benzoylated, and the mixed benzoyl derivatives extracted and hydrolysed. The mixed amino acids obtained were coupled with 1-fluoro-2:4-dinitrobenzene and the glycine derivative separated chromatographically essentially as described by Krol (1952). The ^{15}N-content of the hippuric acid was consistently between two and three times greater than the ^{15}N-content of the corresponding uncombined urinary glycine. This result could arise if the glycine moeity of hippuric acid were derived, in part at least, preferentially from dietary glycine before the latter had become uniformly distributed in the free-glycine metabolic pool.

We are using the uncombined urinary glycine as a means of investigating the free glycine metabolic pool size and turnover rate and suggest preliminary figures of 6·3 g. (≡0·12 mmol./100 g. body weight) and 1·5 g./hr. (≡0·68 mmol./day/100 g. body weight) for the pool size and turnover rate in a 70 kg. adult respectively. The former result, related to 100 g. body weight is similar to the value (about 0·13 mmol./100 g.) obtained by Arnstein & Neuberger (1951) in a different type of experiment for what they termed 'the first glycine metabolic pool' in the rat.

We are pleased to acknowledge our indebtedness to Professor A. Wormall, F.R.S., for the provision of facilities for the mass spectrometric analyses.

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Ethylmercapturic Acid and its Formation *in vivo*. By A. E. R. Thomson, G. A. Maw and L. Young. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

It has been reported by Roberts & Warwick (1957) that the administration of ethyl methanesulphonate, $CH_3 \cdot SO_2 \cdot O \cdot C_2H_5$, to rats is followed by the excretion of two sulphur-containing acids, one of which is ethylmercapturic acid (*N*-acetyl-*S*-ethyl-L-cysteine) and the other possibly the corresponding α-keto acid. In the present work we have obtained evidence that the administration of bromoethane to rats leads to the excretion of ethylmercapturic acid in the urine:

$$C_2H_5Br \rightarrow C_2H_5 \cdot S \cdot CH_2 \cdot CH(NH \cdot CO \cdot CH_3) \cdot CO_2H$$

Our observations, together with those described in the following paper by Bray & James (1958), suggest that mercapturic acid formation may be a general metabolic reaction for alkyl halides.

S-Ethyl-L-cysteine was prepared by the ethylation of L-cysteine in anhydrous ammonia with iodoethane and was converted to ethylmercapturic acid by the action of ketene. The mercapturic acid was also prepared by the ethylation of *N*-acetyl-L-cysteine in anhydrous ammonia. The synthetic products were used as reference compounds in studies of the urine of rats dosed with bromoethane by subcutaneous injection (1·25 g. C_2H_5Br/kg. body wt. as a 40% (w/v) solution in arachis oil). Examination of the urine by paper chromatography using three different solvent systems indicated the presence of ethylmercapturic acid, and chloroform extracts of the acidified urine were also found to contain the compound. Ethylmercapturic acid was detected by spraying the chromatograms with 0·1 M-$K_2Cr_2O_7$–acetic acid (1:1) and then with 0·1 M-$AgNO_3$, as was used by Knight & Young (1957) for the detection of premercapturic acids and mercapturic acids. Paper chromatography also showed that ethylmercapturic acid disappeared from the urine when it was hydrolysed with hot HCl, and that this was accompanied by the appearance of *S*-ethyl-L-cysteine.

Radiochromatographic studies were made on the urine of rats which had been maintained on a diet containing yeast proteins in which the sulphur-containing amino acids were labelled with ^{35}S. The administration of bromoethane to these animals was followed by the appearance on the chromatograms of two radioactive spots. One of these has not been identified and the other corresponded to ethylmercapturic acid. The mercapturic acid spot was absent from chromatograms of the acid-hydrolysed urine, which did, however, show a radioactive spot corresponding to *S*-ethyl-L-cysteine.

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The Formation of Mercapturic Acids from Aliphatic Compounds *in vivo*. By H. G. Bray and Sybil P. James. (*Physiology Department, Medical School, University of Birmingham*)

Until recently all the mercapturic acids reported in the literature had their acetylcysteyl radical attached either directly to an aromatic nucleus (e.g. *N*-acetyl-*S*-(4-chlorophenyl)-L-cysteine, *N*-acetyl-*S*-(1-naphthyl)-L-cysteine, *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine) or at the end of a side chain attached to an aromatic nucleus (e.g. *N*-acetyl-*S*-benzyl-L-cysteine). Roberts & Warwick (1957) reported the excretion by rats of *N*-acetyl-*S*-ethyl-L-cysteine after administration of ethyl methanesulphonate and Thomson, Maw & Young (1958) now report that this mercapturic acid can be formed by rats from bromoethane. We have obtained evidence that the rabbit excretes mercapturic acids after the administration of 1-bromobutane, -pentane, -hexane, -heptane and octane. These are believed to be formed, in effect, by acetylcysteyl debromination, e.g. the product from 1-bromobutane is *N*-acetyl-*S*-butyl-L-cysteine. The percentage of the dose excreted as mercapturic acid decreases as the chain length increases. Evidence has also been obtained that mercapturic acids are formed from 2-bromobutane and bromocyclohexane and that small amounts of mercapturic acid may be formed, presumably by acetylcysteyl denitration, from 1-nitroethane and 1-nitropropane. The formation of mercapturic acid thus appears to be a general metabolic reaction of certain types of purely aliphatic compounds as well as of aromatic compounds.

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Influence of Insulin and Adrenaline on the Incorporation of [^{14}C]Glucose into Free and Fixed Glycogen Fractions in the Isolated Diaphragm. By Adrienne J. M. Kits van Heijningen and W. A. Bosch. (*Pharmaco-therapeutic Laboratory, University of Amsterdam, Holland*)

In the isolated diaphragm, incubated with glucose, insulin produces an increase in total glycogen (Gemmill, 1940), which is entirely accounted for by an increase in the free fraction (Kits van Heijningen, 1957). To elucidate whether the extra glucose is incorporated into the free fraction directly or via the fixed glycogen, diaphragms were incubated in media containing [U-^{14}C]glucose with and without insulin. Without insulin specific activities (S.A.) after 45 min. were $2{\cdot}20 \pm 0{\cdot}56$ c/μg./min. for free and $2{\cdot}55 \pm 0{\cdot}66$ for fixed glycogen, their rates of increase between 45 and 75 min. running parallel to give final values of $3{\cdot}74 \pm 0{\cdot}67$ and $4{\cdot}02 \pm 0{\cdot}45$ respectively. Addition of insulin increases the S.A. of free glycogen to $6{\cdot}23 \pm 1{\cdot}07$ after 45 min., while no further increase in S.A. occurs between 45 and 75 min. After 35 min., when the increase in free glycogen content has not yet reached its maximum, the S.A. is already 6·24. In the fixed glycogen insulin increases the S.A. only to 3·75 after 45 min., and neither the amount present nor the increase in S.A. between 45 and 75 min. deviate from the control values. Although no figures for the period between 0 and 35 min. could be obtained, the results are not suggestive of an indirect transfer of the extra glucose incorporated, not even if one takes into account that the outer branches of the tree-like glycogen molecule (Cori & Larner, 1951) are metabolically more active than the inner stem (Stetten, Katzen & Stetten, 1956).

If the free fraction serves to replete the fixed when glycogen stores are being drawn upon, the transfer of free to fixed glycogen should, after incubation with insulin, result in an increase in S.A. of the fixed fraction. Therefore, to induce glycogen breakdown, diaphragms preincubated in media containing [^{14}C]glucose and insulin, were transferred to media containing adrenaline and inactive glucose or no glucose at all. Unexpectedly, however, it appeared that adrenaline will no longer cause a decrease in fixed glycogen when the diaphragm was pretreated with insulin. Yet the S.A. of this fraction decreases even if there is no glucose in the medium containing adrenaline. The possible significance of these findings will be discussed.

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Identification of Amino Sugars. By M. J. CRUMPTON. (*Microbiological Research Establishment, Porton, Wiltshire*)

The behaviour of amino sugars has been examined by chromatography on columns of Zeo-Karb 225 resin eluted with 0·33N-HCl by a modification (Rondle & Morgan, 1955*a*) of the method of Gardell (1953). Samples (200 μg.) of each sugar were examined alone and with added glucosamine; the glucosamine emerged in a peak position (ml. effluent) which was reproducible for a particular column provided that HCl of the same normality was used.

Amounts of eluted sugar were estimated by the procedure of Rondle & Morgan (1955*b*). Sugars producing chromophores whose absorption maxima were identical with that of glucosamine (530 mμ) were estimated using glucosamine standards. 'Muramic acid' (Strange, 1956; Kent, 1957) and D-fructosamine (Maurer & Schiedt, 1935) however, gave absorption spectra with maxima at 505 and 550 mμ respectively; these sugars were therefore estimated using standards of the same sugar, colour intensities being measured at the wavelength of maximum absorption. The absorption spectrum produced by the sugar eluted from the column in its peak position was measured, since in this way muramic acid and fructosamine could be differentiated from other amino sugars. The peak volume (ml. effluent) in which the sugar emerged was determined and has been expressed relative to that of glucosamine. The resultant ratio ('$R_{glucosamine}$ value') is independent of column length, amount of resin and the normality of the acid used for elution, at least within the range 0·25–0·50N. This property together with the absorption maximum of the chromophore are of use for the identification of amino sugars.

Equal weights of amino sugars (μg. as base) produced in the Rondle & Morgan (1955*b*) test different colour intensities at 530 mμ. The amount of colour formed has been determined relative to that produced by an equal weight of glucosamine; these figures (% ± 3 %) are given in parenthesis following the $R_{glucosamine}$ values (± 0·01) estimated from the column data: D-glucosamine uronic acid (Heyns & Paulsen, 1955), 0·71 (100); a synthetic stereoisomer of muramic acid (Kent & Strange, 1958), 0·87; D-mannosamine (Kuhn & Bister, 1957), 1·07 (69) (cf. Comb & Roseman, 1958); muramic acid, 1·10 (27); D-galactosamine, 1·20; D-gulosamine (Tamelen, Dyer, Carter, Pierce & Daniels, 1956), 1·21 (80); D-allosamine (from *N*-acetyl-D-allosamine; Jeanloz, 1957), 1·24 (83); D-xylosamine (from *N*-acetyl-D-xylosamine; Wolfrom & Anno, 1953), 1·41; D-fructosamine, 1·52 (131); D-talosamine, 1·60 (112); D-fucosamine (Crumpton & Davies, 1956), 1·94 (94).

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The Effect of Haemorrhage on the Incorporation of [α-^{14}C]Glycine into Stercobilin. By C. H. GRAY and J. J. SCOTT. (*Departments of Chemical Pathology, King's College Hospital and St Mary's Hospital Medical Schools, London*)

When [α-^{14}C]glycine is fed to a healthy man, the resulting radioactivity of the faecal stercobilin shows two peaks, one at 115 days corresponding to the maximum rate of breakdown of the labelled red cells and one during the first 7 days. That fraction responsible for the first peak is here termed 'early' stercobilin. It has been suggested that it may arise in the bone marrow by premature breakdown of haemoglobin from intact red cells (London, Shemin, West & Rittenberg, 1949; Gray, Neuberger & Sneath, 1950) or from haem in excess of globin (Neuberger, Muir & Gray, 1950).

Formation other than from haemoglobin has been considered (London, West, Shemin & Rittenberg, 1950); evidence was obtained (Berlin, Neuberger & Scott, 1956) that some δ-aminolaevulic acid is converted rapidly to stercobilin in man, haemoglobin not being an intermediate.

If formation of 'early' stercobilin is related to haemopoiesis there should be proportionality between haemopoietic activity of the marrow and the amount of radioactivity in 'early' stercobilin. The incorporation of [α-^{14}C]glycine into this fraction has therefore been studied before and after haemopoiesis had been stimulated by bleeding.

[α-^{14}C]Glycine was given orally on two occasions to a man of 53 years having very mild photosensitivity and slightly raised excretion of coproporphyrin; after each occasion faecal stercobilin was isolated at convenient intervals over 17 days. Four days before the second dose of [α-^{14}C]glycine, blood (1 l.) was removed, so that the isotope was administered when the response to haemorrhage was near its height, as judged by the reticulocyte count of the circulating blood. The findings to be presented show that the 'early' stercobilin was related to the rate of red cell production since the activity of stercobilin formed after haemorrhage was invariably greater than that formed in the corresponding period before haemorrhage. Between the 5th and 8th days, when the difference was greatest, the specific activities differed by a factor of 3·4. Throughout the 5 weeks of investigations the faecal excretion of coproporphyrin and protoporphyrin remained unchanged, averaging 1·8 and 0·5 mg./day respectively.

In addition to the findings obtained as a result of the removal of blood it is also clear, from comparison of the activities during the early maximum between days 2 and 5 and the later maximum at 115 days, that there was no significant increase in the amount of 'early' stercobilin relative to late-labelled stercobilin such as occurs in congenital porphyria.

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The Separation of Monosaccharides on Charcoal-Celite Columns. By J. S. D. Bacon. (*Department of Biochemistry, Macaulay Institute, Aberdeen*)

Chromatography on activated charcoal-Celite columns (Whistler & Durso, 1950) has been applied very widely to the separation of oligosaccharides, particularly di- and trisaccharides, but monosaccharides are retained only slightly by the charcoals commonly used, and have usually been separated by partition chromatography on cellulose powder (Hough, Jones & Wadman, 1949) or Celite (Lemieux, Bishop & Pelletier, 1956).

Following an observation by Dr R. Côté, Dr W. J. Whelan has found an activated charcoal (Ultrasorb S.C. 120/40) which retains monosaccharides to a useful extent, and can thus be used to separate them from inorganic salts (Hughes & Whelan, 1958). Dr M. J. Clancy in the same laboratory has developed the use of columns of this charcoal in a new preparative method for the isolation of pure L-fucose from its mixture with other monosaccharides in acid hydrolysates of *Ascophyllum nodosum*.

We also have examined the possibility of using columns of Ultrasorb-Celite to separate mixtures of monosaccharides. Of xylose, ribose, glucose, fructose, fucose and rhamnose the pentoses are least, and the 6-deoxyhexoses most, strongly adsorbed. 'Tailing' is noticeable even when gradient elution is employed. The useful capacity of the columns is low and the concentration of sugar in the effluent is barely sufficient for direct examination by paper chromatography. Examination of several batches of Ultrasorb (kindly supplied by British Carbo Norit Union Ltd., London Road, West Thurrock, Grays, Essex) showed no marked variation in its capacity to retain monosaccharides.

When 25 mg. quantities of xylose, glucose and rhamnose were applied to a column of 20 g. Ultrasorb:20 g. Celite No. 535 and eluted by a gradient produced by dropping 4% (v/v) ethanol into 500 ml. water (cf. Bacon & Bell, 1953) the sugars were detectable in the effluent as follows (mean of 5 experiments): xylose 315–440 ml., glucose 355–520 ml., rhamnose 505–650 ml.; the corresponding figures for a B.D.H. activated charcoal-Celite column were: 126–194, 134–208, 208–272 ml.

The value of this method lies in its use in conjunction with partition chromatography; it should be particularly useful for separating pairs of sugars, e.g. ribose-fucose, which have very similar R_F values in aqueous butanol.

I am very grateful to Dr Whelan and his colleagues at the Lister Institute for information about the charcoal and their unpublished experiments. This work was done with the technical assistance of Miss A. M. Joss.

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Conductivity and pH Measurements in Protein Electrophoresis Experiments. By M. A. Rosemeyer, E. M. Shooter and E. R. Skinner. (*Physics Department, Birkbeck College and Department of Biochemistry, University College London*)

The spread of a moving protein boundary in electrophoresis is controlled by the conductivity and pH changes across this boundary (Longsworth, 1947) and the magnitude and direction of these phenomena are dependent on the relative equivalent concentrations of buffer salt and protein, and on buffer ionization and ion mobility. The effect of these variables on the spread and therefore resolution of the boundaries of two closely related proteins, haemoglobins A and S, has already been examined (Shooter & Skinner, 1955).

Further information has now been obtained by determining the conductivities and pH's of the phases separating in the ascending limb during the analysis of these proteins. Four different systems, phosphate and cacodylate buffers below the isoelectric point of the haemoglobins and barbiturate and tris(hydroxymethyl)aminomethane buffers above the isoelectric point, all at constant ionic strength and equivalent concentration of protein, have been examined. In all systems the experimentally determined sharpening ratio (Dismukes & Alberty, 1954) correlated well with the actual spread of the moving boundaries. Where the ratio was high, as in phosphate buffer, good resolution was obtained and conversely, where the ratio was close to unity as in barbiturate buffer, practically no resolution was achieved.

Some unusual features appear in these experiments. The relative magnitude of the conductivity changes in the various buffers do not agree with the Dole (1945) theory. Furthermore, although the pH changes across the protein boundaries are in the predicted direction they are, with the possible exception of the experiment in tris buffer, greater in magnitude. A noticeable characteristic of three of these experiments is the large pH difference between the dialysed protein and buffer solutions. Similar results have been found in other protein systems, for example, ribonuclease in phosphate or bovine serum albumin in chloride solutions. It is significant, however, that for ovalbumin in acetate buffer, where the electrophoretic behaviour can be accurately predicted by the Dole theory, only small changes of pH occur on dialysis.

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The Electrophoretic Analysis of Human Haemoglobins. By E. M. Shooter, E. R. Skinner and J. C. White. (*Department of Biochemistry, University College London, and Department of Pathology (Haematology), Postgraduate Medical School of London*)

One of the buffers (phosphate, pH 6·40, ionic strength 0·04) previously advocated for the analysis of haemoglobins (Shooter & Skinner, 1955) has now been extensively used in the analysis of a variety of haemoglobins. This work illustrates the advantages and disadvantages of using a buffer which causes sharpening of ascending moving boundaries in systems of closely related proteins.

Complete resolution of haemoglobin boundaries is obtained for heterozygous C, E, S, G, J and H haemoglobins and sickle cell-haemoglobin C disease. A necessary consequence of achieving resolution of haemoglobins with very close mobilities, e.g. S and C, is the accentuation of the apparent proportion of the leading component and diminution of the slower component (Hoch, 1950).

Thus, in the analysis of normal adult haemolysates, haemoglobin-A_2 (Kunkel & Wallenius, 1955), which migrates faster than haemoglobin-A, is readily observed, but the slower minor component is more difficult to detect. The elevation of haemoglobin-A_2 in thalassaemia minor, and the appearance of two components in approximately the haemoglobin-A_2 position in stored concentrated oxyhaemoglobin solutions, can also be demonstrated when these are analysed as carboxyhaemoglobin.

Identification by absolute mobilities is possible only with homozygotes since the boundaries due to the two haemoglobins in heterozygotes do not separate in the descending limb. Apparent mobilities from the sharper ascending boundaries can, however, be determined with accuracy. Furthermore, the differences in apparent mobility of the various haemoglobins can be increased by suitable choice of buffer, phosphate giving a greater difference than cacodylate (Itano, 1953).

In mixtures, however, the apparent mobility of any haemoglobin depends both on the type of accompanying haemoglobin and on their relative proportions, and thus identification by mobility differences is often not possible. For this reason, identification of haemoglobins of heterozygotes is best accomplished by addition of a known haemoglobin, and the requisite analysis of three-component mixtures, for example A+S+E, A+E+C and A+S+C, can be achieved in phosphate buffer. Such experiments lead to a relative order of mobilities C>E>S>G>A>J, with H migrating in the opposite direction.

One of us (E.R.S.) is indebted to the Colonial Medical Research Council for a research grant. Part of the apparatus used in this research was purchased with a grant from the Central Research Fund. We wish to thank Drs N. A. Barnicot, J. Garlick, E. Huehns and H. Lehmann for samples of abnormal haemoglobins.

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An Electrophoretic Investigation of Ribonuclease. By M. A. Rosemeyer and E. M. Shooter. (*Physics Department, Birkbeck College and Department of Biochemistry, University College London*)

There is evidence from chromatographic and zone electrophoresis techniques that ribonuclease contains several protein components, two of which are known as ribonuclease A and B (Hirs, Moore & Stein, 1953; Raacke, 1956; Hakim, 1956). This heterogeneity has not been detected in moving boundary electrophoresis experiments. However, it is possible to show that suitable selection of buffer conditions permits the separation of four components by this technique. These experiments fall into two groups; one in which boundary anomalies are suppressed, and a second where these anomalies are accentuated.

The first condition, for example in tris(hydroxymethyl)aminomethane buffers at pH values near the isoelectric point of ribonuclease, at an ionic strength of 0·1, is favourable for the separation of a slow minor component. The second condition holds in sodium acetate buffers near pH 5, ionic strength of 0·06, and gives good resolution of a fast minor component. Experiments at intermediate conditions, for example, in cacodylate buffers at pH 6, ionic strength 0·06, show the presence of a fourth component after electrophoresis for 48 hr. at a field strength of 5·5 v/cm.

Mobilities calculated from both limbs of the Tiselius cell show a good correlation with each other, and are also approximately related to the titration data (Tanford & Hauenstein, 1956). However, this is not true in phosphate buffers (Crestfield & Allen, 1954), which indicates interaction between these ions and the protein.

The difference between the mobility of ribonuclease in phosphate and in other buffers reaches a maximum at pH 6·5–7·0, at 0·06 ionic strength. This indicates that the phosphate–protein interaction is a maximum in this pH range, and suggests that there is a specific association between at least one histidine residue on the protein and these ions. However, the participation of other charged groups is also probable from the mobility differences found at higher pH's.

The considerations are of importance for the action of ribonuclease as a phosphorylase (cf. Weil & Seibles, 1955; Anfinsen & Redfield, 1956). The nature of these interactions, and their quantitative aspects are being further investigated.

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The Fermentation of Lactate and Acrylate by Cell-Free Extracts of the Rumen Organism LC1. By J. N. Ladd and D. J. Walker (introduced by S. R. Elsden). (*A.R.C. Unit for Microbiology, Department of Microbiology, Sheffield University*)

Cell-free extracts of the rumen organism LC1 (Elsden, Volcani, Gilchrist & Lewis, 1956) have been prepared which ferment lactate and acrylate to H_2, CO_2, acetate and propionate, and pyruvate to H_2, CO_2 and acetate. The rate of H_2 production is a convenient measure of the rate of fermentation of these substrates. Freshly prepared extracts fermented both lactate and acrylate rapidly. Extracts which had been dialysed for 4–6 hr. at 4° no longer fermented these two substrates, although they still attacked pyruvate rapidly. The activity of these dialysed extracts was restored by the addition of catalytic amounts (0·8 μmoles/ml.) of either pyruvate, acetyl-phosphate, ATP or ADP; AMP was inactive. Further, lactate catalysed the fermentation of acrylate, and acrylate catalysed the fermentation of lactate.

Extracts which had been dialysed overnight at 4° were not reactivated by catalytic amounts of any of the above compounds. However, when catalytic amounts of acrylate were added to substrate amounts of lactate, H_2, approximately equivalent to the amount of acrylate added, was evolved. A similar effect was observed when catalytic amounts of lactate were incubated with excess acrylate.

There was no loss of activity when extracts were centrifuged for 1 hr. at 144 000 *g* at 2°. The fermentation of lactate by such centrifuged extracts was completely inhibited by $2 \cdot 10^{-5}$ M-2:4-dinitrophenol, $2 \cdot 10^{-3}$ M-azide, 10^{-4} M-hydroxylamine and $2 \cdot 10^{-3}$ M-arsenate. In parallel experiments acrylate fermentation was completely inhibited by 10^{-5} M-2:4-dinitrophenol, $2 \cdot 10^{-4}$ M-azide, $2 \cdot 10^{-5}$ M-hydroxylamine and 10^{-2} M-arsenate.

When [2-^{14}C]lactate was added to an extract and the fermentation stopped by the addition of hydroxylamine, chromatography of the reaction mixture followed by radioautography showed radioactive areas corresponding to acetyl, propionyl and lactyl hydroxamates; in addition, spots appeared on the radioautogram which were not hydroxamates and which have not been identified.

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A Coupled Oxidation-Reduction Reaction Involving Lactate and Acrylate. By D. J. Walker and J. N. Ladd (introduced by S. R. Elsden). (*A.R.C. Unit for Microbiology, Department of Microbiology, University of Sheffield*)

Ladd & Walker (1958) reported that overnight dialysis of LC1 extracts resulted in the complete loss of ability to ferment either lactate or acrylate, whilst hydrogen production from pyruvate remained relatively unimpared. However, when such extracts were incubated in phosphate buffer with lactate and acrylate together, hydrogen was produced; the amount was approximately equal to the amount of the substrate which was limiting. Thus, when 5, 10 or 20 μmoles of acrylate were incubated with an excess of lactate, 4·0, 7·8 and 15·5 μmoles of H_2 were produced. When the same amounts of lactate were incubated with excess acrylate, 4·4, 9·1 and 17·6 μmoles of H_2 were formed.

These observations suggest that extracts catalyse a reaction between lactate and acrylate yielding pyruvate and propionate; the pyruvate so formed is then broken down to H_2, CO_2 and acetate. This hypothesis requires that the pyruvate is derived from lactate and the propionate from acrylate. This was tested by incubating an excess of [2-^{14}C]-lactate with acrylate and examining the products. A carbon and hydrogen balance showed that equal amounts of H_2, CO_2, propionate and acetate were formed. The isotope was located almost entirely in the acetate, which had a specific activity similar to that of the lactate added. In parallel experiments in which imidazole buffer was used instead of phosphate buffer, a large amount of

pyruvate accumulated which had the same specific activity as the lactate. In neither case was propionate significantly labelled.

This reaction was also demonstrated in non-dialysed extracts when the following inhibitors of lactate and acrylate fermentation were present: 10^{-4}M-hydroxylamine, 2×10^{-3}M-azide or 10^{-4}M-dinitrophenol.

The results given in both this and the previous communication suggest that the fermentation of both lactate and acrylate can be explained by the following equations:

$$\text{lactate} \rightleftharpoons \text{acrylate} \quad (1)$$

$$\text{lactate} + \text{acrylate} \rightarrow \text{pyruvate} + \text{propionate} \quad (2)$$

$$\text{pyruvate} + H_2O \rightarrow \text{acetate} + H_2 + CO_2 \quad (3)$$

The mechanism of reaction (1) is unknown and would seem likely to be complex. The products of fermentation can be accounted for by reactions (2) and (3), which, so far as is known, proceed without activation. Thus, it is reasonable to suppose that reaction (1) is the step which requires activated substrates. Further, because neither reaction (2) nor (3) are inhibited by hydroxylamine, azide or dinitrophenol, these compounds must inhibit some step in reaction (1).

This work was supported by the Rockefeller Foundation.

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Changes in Retinal Glycolysis Co-incident with the Opening of the Eye in the Rat. By C. N. Graymore (introduced by M. Kerly). (*Department of Pathology, Institute of Ophthalmology, University of London*)

The anaerobic glycolytic activity of the rat retina doubles during the 10th–13th day after birth (Graymore, 1958*a*), this surge in activity coinciding with the period in which the eyes open. In the rats used in this investigation the eyes opened on the 12th–13th day of life, and it was suggested that the observed increase may be light-dependent, possibly as a response to an increased energy demand co-incident with the appearance of electrical activity in the retina.

The present communication describes experiments designed to test this hypothesis. Three litters of albino rats, of the same strain as those used in the above experiments, were kept in total darkness between the 10th and 15th days of life. At the end of this period they were sacrificed by decapitation in the dark and their retinae removed in the light by the procedure reported previously (Graymore, 1958*b*). The rate of anaerobic glycolysis was determined manometrically in a Krebs–Ringer bicarbonate buffer gassed with 95% N_2–5% CO_2, and the final result expressed on a protein basis. It was found that these animals showed the same increase in activity during their confinement as those bred normally in the light, and it would appear therefore that the increased activity is independent of light stimulation, possibly being a reflection of the state of histological development of the retina.

Furthermore, one exceptional litter was obtained in which only three of the animals had opened their eyes by the 14th day of life. The eyes of the remainder were still firmly closed. Examination showed that the animals which had their eyes closed exhibited the same high retinal glycolytic rate as those in which the eyes were open. This confirms the above observations.

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The Effects of Thiopentone, Azacyclonol (Frenquel) and Reserpine on Rat Brain Phospholipid Metabolism *in vivo*. By G. B. Ansell and Anna Morgan. (*M.R.C. Neuropsychiatric Research Unit, Whitchurch Hospital, Cardiff*)

It is known that the rate of phospholipid synthesis in brain tissue is depressed in certain conditions, e.g. insulin hypoglycaemia and thiopentone anaesthesia (Dawson & Richter, 1950; Ansell & Dohmen, 1957). These studies have now been extended to show the effect of certain compounds on the turnover of two known phospholipid precursors, namely phosphorylethanolamine and phosphorylcholine.

The phospholipid turnover was measured by the uptake of [^{32}P]orthophosphate into individual phospholipid fractions after a 3 hr. isotope exchange period, using the method of Dawson (1954). For the measurement of the uptake of ^{32}P

into the free phosphate esters of the tissue extracts, the following procedure was used. Trichloroacetic acid extracts of brain tissue were freed from nucleotides by adsorption onto charcoal. Most of the inorganic phosphate was then removed with barium hydroxide and the barium salts of the remaining phosphate esters were converted to ammonium salts with Amberlite IR-120 (NH_4^+) (cf. Heald, 1956). Phosphorylethanolamine and phosphorylcholine were then separated by paper chromatography.

The uptake of ^{32}P into rat brain phosphorylcholine after a 3 hr. exchange period was approximately twice that into phosphorylethanolamine, which may be related to the fact that the synthesis of phosphatidyl choline in the brain proceeds more rapidly than that of the ethanolamine-containing phospholipids (Ansell & Dohmen, 1957). Thiopentone anaesthesia had no significant effect on the synthesis of the precursor phosphorylethanolamine or phosphorylcholine, whereas it decreased by 50% the incorporation of labelled phosphate into the phospholipids phosphatidyl ethanolamine and phosphatidyl choline. This indicates that thiopentone may affect steps in the synthetic chain other than the simple phosphorylation of lipid bases.

Magee, Berry & Rossiter (1956) have observed an effect of azacyclonol on phospholipid labelling by ^{32}P *in vitro*. In our experiments doses of azacyclonol of the order of 70 mg./kg. body weight had no apparent effect on the metabolism of phospholipids, but larger doses (200–250 mg./kg. body weight) depressed the synthesis; no effect was, however, observed on the synthesis of phosphorylethanolamine and phosphorylcholine. Reserpine (5–10 mg./kg. body weight) had neither a depressant effect on synthesis of phospholipid nor on the synthesis of the two lipid bases studied. In these experiments the anaesthetic and tranquillizer drugs appear to act at different points in the synthetic chain.

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The Production of Lipolytic Activity in the Circulation of the Hind Limb in Response to Heparin. By D. S. Robinson and P. M. Harris. (*Sir William Dunn School of Pathology, Oxford*)

It has been shown that lipolytic activity can be demonstrated in the blood following the intravenous injection of heparin. No such activity can be demonstrated when heparin is added to blood *in vitro*. This suggests that some factor from the tissues may be necessary for the production of activity (Robinson & French, 1957). In the present communication some experiments on the lipolytic activity which is induced in the circulation of the hind limb in response to the injection of heparin will be described.

Two series of experiments were performed. In the first heparin was injected into the femoral arteries of rabbits and serial blood samples were taken from their femoral veins from the time of injection. Lipolytic activity was present in the venous blood about 20 sec. after the injection of heparin into the artery. When Evans Blue dye was injected with the heparin, the dye and the lipolytic activity appeared in the same sample of venous blood. Lipolytic activity persisted in the venous blood for some time after the dye concentration had fallen to a low level. These experiments, together with others already reported (Robinson, Harris & Ricketts, 1958), suggest that the lipase, or some substance essential for the lipase action, may be removed by the circulating heparin from the inner surface of the blood-vessel wall. The finding that lipolytic activity can be produced in the blood circulating through the hind limb suggests that this response to heparin may be a general characteristic of blood vessels.

In a second series of experiments the isolated hind-quarters of rats were perfused with heparin in plasma, in a solution of albumin, or in various mixtures of plasma and albumin solution. The lipolytic activity detectable in the perfusate fell as the proportion of plasma in the perfusing fluid was lowered. Plasma added to the perfusate before its lipolytic activity was determined did not raise that activity. These experiments suggest that the appearance of lipolytic activity in blood in response to heparin injection is dependent on the presence of some component of the plasma.

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Effect of Cyanide on Succinic Dehydrogenase. By D. KEILIN and TSOO E. KING.* (*Molteno Institute, University of Cambridge*)

It was shown by Tsou (1951) that the oxidation of succinate by heart-muscle preparation with methylene blue as hydrogen acceptor is abolished by prolonged incubation of the preparation with cyanide. While we confirmed this observation, we found that the activity of the same cyanide-treated preparation, when tested with phenazine methosulphate, was if anything higher than that of the untreated preparation. A similar result was obtained with soluble succinic dehydrogenase prepared by the method of Wang, Tsou & Wang (1956) slightly modified by us (Keilin & King, 1958). While succinic dehydrogenase reacts directly with phenazine (Kearney & Singer, 1956), with methylene blue it reacts through some components of the respiratory chain (Slater, 1949). Since the oxidation of succinate by the heart-muscle preparation pre-incubated with cyanide showed no inhibition when tested with phenazine, while it showed a complete inhibition when tested with methylene blue, it suggested at first that cyanide reacted not with dehydrogenase but with another component of the respiratory chain. That this is not so can be demonstrated as follows: It was previously shown (Keilin & King, 1958) that on bringing together soluble succinic dehydrogenase and a particulate heart-muscle preparation containing the cytochrome oxidase system, but devoid of succinic dehydrogenase, an active succinic oxidase system was obtained which catalysed the oxidation of succinate with either methylene blue or oxygen. However, such reconstitution of the succinic oxidase system could not be obtained by using soluble succinic dehydrogenase either previously incubated with cyanide or isolated from a cyanide-treated heart-muscle preparation. All this indicates that the cyanide inactivation of heart-muscle preparation in Tsou's experiments was due to its reaction with succinic dehydrogenase itself. The fact that the reaction of succinic dehydrogenase with cyanide is slow, complete, but irreversible, militates against its possible reaction with the iron which is supposed to form part of the enzyme. Moreover, since cyanide did not react with flavin it is conceivable that it reacted with the disulphide group of protein to give one sulphydryl and one thiocyanate group (Mauthner, 1912). Whether this –S–S– group is present independently of, or derived from, pre-existing –SH groups of succinic dehydrogenase remains open for further study.

* Senior Post-doctoral Fellow, National Science Foundation and Guggenheim Fellow.

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The Proteolysis of Glucagon and other Peptides by the Rat Liver *in vitro*. By A. J. KENNY. (*Department of Biochemistry, University of Cambridge*)

Glucagon loses biological activity when incubated with homogenates of many tissues. The properties of the enzyme in homogenates of rabbit and rat livers have been previously reported (Kenny, 1956). The proteolysis of glucagon by the supernatant fraction of a rat liver homogenate has been studied by a technique requiring only small quantities of substrate. The incubation mixtures were subjected to paper chromatography with 2-butanol–acetic acid (Light & Simpson, 1956) and the proteolysis of glucagon revealed by the ninhydrin reaction.

The proteolytic enzyme was localized in the supernatant fraction of the rat liver homogenate. The pH optimum was about 7·5. The enzyme was strongly inhibited by *p*-chloromercuribenzoate and other inhibitors of thiol enzymes. The presence of insulin and corticotropin also inhibited the proteolysis of glucagon. In these respects the proteolytic enzyme was similar to the enzyme which causes biological inactivation. In addition, proteolysis of glucagon was partially inhibited by high concentrations of ethylenediaminetetra-acetic acid, tolbutamide, carbutamide and indole-3-acetic acid. Insulin was not hydrolysed under the same conditions as glucagon, although fractions A and B from oxidized insulin (Sanger, 1949) were attacked.

The products resulting from the incubation of glucagon with the supernatant fraction from liver homogenate were fractionated by paper chromatography and paper electrophoresis. Free amino acids occurred in the digests. No small peptides were found among the products at any time during incubation. Each of the 15 amino acids occurring in glucagon (Bromer, Sinn & Behrens, 1957) were identified as products of hydrolysis. In similar

experiments with fraction A, glycine, isoleucine, valine, glutamine and glutamic acid were identified, together with smaller amounts of cysteic acid, alanine and serine. These amino acids occur at the *N*-terminal sequence of fraction A of beef insulin (Sanger & Thompson, 1953) and the results strongly suggest that an aminopeptidase was responsible. Results with fraction B as a substrate were less conclusive but also consistent with the action of an aminopeptidase. The additional action of a carboxypeptidase could not entirely be ruled out with these substrates, since fractions A and B each contain residues (cysteic acid and proline respectively) near the *C*-termini which might inhibit the progress of hydrolysis by a carboxypeptidase. However, the work of Rademaker & Soons (1957) indicates that carboxypeptidase is absent from the supernatant fraction of a rat liver homogenate.

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Acetate Utilization in the Sheep. By E. F. Annison and D. B. Lindsay. (*A.R.C. Institute of Animal Physiology, Babraham, Cambridge*)

Acetate is recognized to be an important source of energy to ruminants. In these animals dietary carbohydrates are largely fermented to volatile fatty acids in the rumen, and acetic acid usually accounts for 60–70 % of the acids present.

The utilization of acetate in the sheep has been investigated using [1-^{14}C]acetate and a constant infusion technique similar to that used by Steel, Wall, de Bodo & Altszuler (1956) for glucose. Labelled acetate was infused at a constant rate until the specific activities of plasma acetate and respired CO_2 were constant. The quantity of carrier acetate infused was insufficient to affect the circulating levels of acetate. Sheep fasted for 24 hr. were employed to minimize CO_2 production in the rumen, and to eliminate rapid changes in ruminal acetate production which occur after feeding. The relative contributions of respiratory CO_2 and ruminal CO_2 to the total CO_2 production were checked by using a face mask for total CO_2 collection, and tracheal incubation which allowed only respiratory CO_2 to be collected.

Preliminary results indicated that at plasma concentrations of 0·4–0·5 m-mole/l. acetate utilization rates were 1·4–2·1 m-moles/hr./kg. body wt. Utilization rates do not distinguish between acetate immediately oxidized and that used for synthesis. The ratio of the specific activities of circulating acetate and respired CO_2 under equilibrium conditions, showed that about 25 % of the CO_2 was derived from acetate. Measurement of total CO_2 production allowed the percentage of the acetate utilized which appeared as CO_2 to be calculated, and values of 45–50 % were obtained.

In single injection experiments using 100 μc of [1-^{14}C]acetate in fasting sheep the labelled material rapidly disappeared from the circulation with a half-life of 3·5–4 min. Respired CO_2 reached maximum specific activity after 12 min. and declined to half this value in about 2 hr. Of the total CO_2 expired in 24 hr. after injection, about 50 % appeared within the first 2 hr.

Further experiments are in progress to investigate acetate utilization rates at enhanced concentrations of acetate. The constant infusion method is particularly suited to this purpose since the plasma concentrations can be adjusted to any desired level. Similarly, the influence of raised blood levels of other metabolites, such as glucose can be studied.

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DEMONSTRATIONS

The Purification of Proteins using Zone Electrophoresis on Plastic Sponge. By E. D. Wills. (*Department of Biochemistry, The Medical College of St Bartholomew's Hospital, London, E.C.* 1)

A Micro-Reaction Calorimeter. By J. H. Ashby, S. P. Datta and J. J. Martin. (*Department of Biochemistry, University College, London, W.C.* 1)

Quantification in Agar Gel Diffusion Plates. By J. G. Feinberg. (*Bencard Allergy Research Unit, Beecham Research Laboratories, Betchworth, Surrey*)

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **373rd Meeting** *of the Biochemical Society was held in the Department of Biochemistry of the University of Edinburgh on Saturday,* 17 *May* 1958, *when the following papers were read:*

COMMUNICATIONS

The Interaction of Zinc, Cadmium and Mercury Ions with Native and Modified Soluble Proteins.

By D. J. Perkins. (*Department of Chemical Pathology, St George's Hospital Medical School, S.W.* 1)

There are a number of enzyme systems in which zinc is essential; however, cadmium is associated with few such systems and mercury is toxic to many. The interactions of these ions with human serum albumin as a model protein have been studied in an attempt to understand these observations.

Equilibrium dialysis experiments with either tracer ^{65}Zn or ^{115}Cd at concentrations of 10 μg./ml. of metal showed that maximum binding was obtained at pH 6·5. One albumin molecule bound either 5·5 atoms of zinc or 2·5 atoms of cadmium. There are sixteen imidazole sites per molecule (Gurd & Goodman, 1952) available to these metal ions at this pH. Chemical modification of the protein by esterification, acetylation, deamination, coupling with glycine, and iodination had no effect on binding. This was expected as these procedures do not effect the imidazole residues.

The difference in binding of zinc and cadmium ions is similar to that found for their peptide complexes (Perkins, 1954). This does not explain the toxicity of cadmium to the whole animal.

Equilibrium dialysis using tracer ^{203}Hg showed a maximum uptake of 21 atoms of metal per molecule of albumin at pH 4. This suggests combination through the free carboxyl groups of the molecule. At more alkaline pH it would still be possible for the metal to co-ordinate with other terminal groupings. Toxicity is probably due to the blocking of almost all 'active centres'.

Modification of albumin with bromoacetic acid (Korman & Clarke, 1956) produced a remarkable increase in metal binding. Maximal binding for each mole of protein was 51 atoms of zinc, or 50 atoms of cadmium, or 125 atoms of mercury. The similarly modified bovine albumin is excreted unchanged, in part at least, by the rat kidney (Korman & Clarke, 1956) and so may prove of use in removing toxic quantities of metal ions from the whole animal.

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Biosynthesis of Polyacetylenic Antibiotics.

By J. D. Bu'Lock and H. Gregory. (*Department of Chemistry, University of Manchester*)

The Basidiomycete B. 841 converts both glucose and ethanol into polyacetylenes, viz. nemotinic acid (I), odyssic acid (II), and their derivatives (Bu'Lock, Jones & Leeming, 1955, 1957). When [1-^{14}C]acetate was added to the glucose medium of replacement cultures (Bu'Lock & Leadbeater, 1956) up to 20% of its radioactivity appeared in these polyacetylenes; the labelled (I) thus produced was converted into γ-undecanolactone. This on alkali fusion afforded mainly octanoic and propionic acids, which were degraded by established methods (Popjak, French, Hunter & Martin, 1951; Hunter & Popjak, 1951; Blomstrand, 1954) to afford individual C atoms as $BaCO_3$. Thick-film counting of the latter established the labelling pattern shown for (I), the 6 atoms marked with an asterisk being uniformly labelled within the experimental error ($\pm 5\%$).

$$6CH_3\cdot CO_2H \rightarrow H\cdot \overset{*}{C}\equiv C\cdot \overset{*}{C}\equiv C\cdot \overset{*}{C}H=C=\overset{*}{C}H\cdot CHOH\cdot \overset{*}{C}H_2\cdot CH_2\cdot \overset{*}{C}O_2H \quad (I)$$

$$CH_3\cdot C\equiv C\cdot C\equiv C\cdot CH=C=CH\cdot CHOH\cdot CH_2\cdot CH_2\cdot CO_2H \quad (II)$$

To account for the observed synthesis of (I) from 6 molecules of acetate requires at least three special reactions. First, the introduction of acetylenic (and equivalent allenic) unsaturation; our work confirms the hypothesis (Bu'Lock, 1956) that

this reaction operates within the context of repeated acyl condensation. Secondly, the introduction of oxygen at $C_{(4)}$, by direct oxidation or by hydration of a double bond. Thirdly, the elimination of a twelfth C atom, derived from acetate-methyl, which in the minor metabolite (II) apparently remains intact; this reaction is perhaps best envisaged as one of ω-oxidation followed by decarboxylation.

The postulates necessitated by our observations are sufficient to account also for the biogenesis of other polyacetylenes (cf. Bu'Lock, 1956, for references). Given a mechanism for the introduction of triple bonds, the derivations from acetate of the acetylenic seed oil acids and insecticidal *N-iso*butylamides, and of the series of C_{10} compounds from Compositae and fungi, present no especial difficulties. The occurrence in fungi of ω-oxidation not followed by decarboxylation is strongly suggested by the co-occurrence of pairs of acetylenic derivatives of decanoic and deca-1:10-dioic acids, whilst the postulated elimination of a terminal methyl group to give compounds with an odd number of C atoms accords with the structures of mycomycin and of other compounds with a terminal $HC{\equiv}C{\cdot}$ group including a series of C_9 polyacetylenes from *Coprinus quadrifidus*, recently characterized by E. R. H. Jones and J. Stephenson (personal communication).

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Mast Cell Granules, Containing 5-Hydroxytryptamine, Histamine and Heparin, Morphologically and Biochemically Distinct from Mitochondria. By R. J. Barrnett, P. Hagen and F. L. Lee. (*Departments of Anatomy and Pharmacology, Harvard Medical School, Boston, Massachusetts*)

The 'large granule' fraction obtained from mouse mast cell tumours (Furth, Hagen & Hirsch, 1957) has been subjected to centrifugation in a density gradient as previously applied to the separation of 'large granules' of adrenal medulla (Blaschko, Hagen & Hagen, 1957). A morphologically pure population of particles was obtained (as determined with the electron microscope) with a specific gravity equal to that of a 2M-sucrose solution. These granules contain most of the histamine, 5-hydroxytryptamine and heparin of the mast cell. On electron-microscopic examination they are seen to have a homogeneous dense interior and a thin outer membrane. The mitochondrial fraction did not sediment below the 1·6M-sucrose layer. When this fraction was viewed with the electron microscope it was seen to consist predominantly of fairly normal as well as distorted mitochondria. This fraction contained the greater part of the fumarase and amine oxidase of the mast cell. Thus the granules bearing the histamine, heparin and 5-hydroxytryptamine are morphologically as well as biochemically distinct from mitochondria.

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Hydrogen Transport in Ox-Adrenocortical Mitochondria in Relation to Steroid Hydroxylation. By J. K. Grant and K. Mongkolkul. (*Department of Biochemistry, University of Edinburgh*)

It has been shown (Grant & Brownie, 1955; Sweat & Lipscomb, 1955) that TPNH is required for the 11β-hydroxylation of steroids by enzymes in ox-adrenocortical mitochondria; and Grant (1956) has demonstrated that the role of fumarate, commonly added to enzyme reaction mixtures for steroid hydroxylation, is to effect TPN^+ reduction according to the reactions fumarate $\rightarrow$ L-malate; L-malate + $TPN^+ \rightarrow$ pyruvate + CO_2 + TPNH + H^+. Citrate was found to be a feeble 'activator' of steroid 11β-hydroxylation (Brownie & Grant, 1954), although its addition to the reaction mixture might be expected to result in reduction of TPN^+ coupled to D-*iso*citrate oxidation. It has now been shown that citrate and *iso*citrate are readily oxidized at approximately equal rates by ox-adreno-

cortical mitochondria under the conditions employed for steroid hydroxylation. Thus it is unlikely that permeability factors (Johnson & Lardy, 1958) or deficiency of aconitase (Dickman & Speyer, 1954) can account for the feeble 'activation' of steroid hydroxylation. The coenzyme specificity of adrenocortical mitochondrial *iso*citric dehydrogenase was therefore investigated using suspensions of the particles depleted of native pyridine nucleotide by ageing. The cytochrome system was blocked by cyanide and 2:6-dichlorophenolindophenol was used as terminal electron acceptor (Ernster & Navazio, 1956). Under these conditions reduction of added DPN^+ or TPN^+ in presence of *iso*citrate may be followed by measuring the decrease in optical density at 600 mμ. The activity of the DPN^+-linked *iso*citric dehydrogenase was found to be greater than that of the TPN^+-linked enzyme, suggesting that in the steroid hydroxylation experiments added citrate provides *iso*citrate which is oxidized predominantly by a DPN^+-linked enzyme, thus failing to produce adequate amounts of TPNH.

It has been suggested by Kaplan, Swartz, Frech & Ciotti (1956) that stimulation of *iso*citrate oxidation by DPN^+ in liver mitochondria is due to oxidation of trace amounts of TPNH by DPN^+, catalysed by a mitochondrial transhydrogenase. We have, however, now shown that oxidation of TPNH by ox-adrenal mitochondria is scarcely affected by DPN^+. Indeed the low activity observed for the transhydrogenase catalysing $TPN^+ + DPNH \rightleftharpoons DPN^+ + TPNH$ suggests that this enzyme may not play an important role in the provision of TPNH for steroid hydroxylation (cf. Sweat & Lipscomb, 1955).

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The Incorporation of [4-^{3}H]Thymidine and [8-^{14}C]Adenine into DNA by Particle-free Extracts of Mammalian Cells. By R. M. S. Smellie, A. Hope McArdle, H. M. Keir and J. N. Davidson. (*Biochemistry Department, The University of Glasgow*)

In previous papers (Smellie, Thomson & Davidson, 1958; Thomson, Smellie & Davidson, 1958) we have discussed the utilization of [^{14}C]formate, [2-^{14}C]-glycine, [8-^{14}C]adenine and [6-^{14}C]orotic acid for the biosynthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) by Ehrlich ascites carcinoma cells *in vitro*. These experiments have been extended to show the ability of such cells to utilize [2-^{14}C]thymine and [4-^{3}H]thymidine for DNA synthesis and [2-^{14}C]uracil for RNA formation.

Completely disrupted preparations of ascites cells have been found capable of incorporating [4-^{3}H]thymidine into DNA, and the particle-free extract obtained by centrifuging these preparations at 105 000 *g* has also been found to incorporate thymidine into DNA. The optimum pH for this reaction has proved to be in the region of 8 and the process requires ATP and DPN in concentrations of at least 2·5 μmoles/ml. Glucose is also required for the incorporation to proceed. Degradation of the radioactive DNA by acid hydrolysis shows the activity to be localized in the thymine, and treatment with deoxyribonuclease results in liberation of acid-soluble fragments containing radioactivity.

This incorporation of tritiated thymidine by cell-free extracts is not inhibited by nitrogen mustard in concentrations of 50 μg./ml. or by 500 r. of X-irradiation (85 kv, 5 ma). In other experiments [8-^{14}C]adenine was incorporated into both DNA and RNA adenine by the extracts under similar conditions.

Extracts prepared from thymocytes by a similar procedure have also proved capable of incorporating [4-^{3}H]thymidine into DNA.

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The Intensive Culture of *Nitrobacter*. By G. W. GOULD and H. LEES. (*Department of Biological Chemistry, University of Aberdeen*)

A perennial problem in working with the nitrite-oxidizing autotroph *Nitrobacter* is to obtain sufficient cell material, the maximum yield being normally about 20 mg. dry weight of cells per litre of culture. We have now found that this yield may be increased either by intense aeration or by removal of the nitrate resulting from nitrite oxidation.

Pure cultures of *Nitrobacter* were prepared from enrichment cultures by successive treatments with terramycin and matromycin followed by dilution almost to extinction. One litre of pure culture, grown according to the method of Lees & Simpson (1957), was used to inoculate 4 l. of sterilized medium in a fully baffled stainless-steel reactor designed on the principles of Chain, Paladino, Ugolini & Callow (1954) and capable of providing conditions of high aeration in 5 l. of culture. Growth of the *Nitrobacter* was measured partly by optical density measurements but chiefly by estimations of the amount of nitrite oxidized. After an initial lag-phase of 10 hr., nitrite oxidation proceeded logarithmically, doubling its rate every 12 hr. until about 500 μg. nitrite-nitrogen/ml. had been oxidized; thereafter the rate of nitrite oxidation was constant. The maximum rate achieved was 25 μg. nitrite-nitrogen oxidized per hour. Oxidation ceased when some 2000 μg. nitrite-nitrogen/ml. had been oxidized and the dry weight of cells was 70 mg./l., a yield much better than normal, but still relatively small. We suspected that cessation of nitrite oxidation (and therefore of cell growth) was due to the accumulation of nitrate, which is known to inhibit nitrite oxidation especially under highly aerobic conditions (Butt & Lees, 1958). We therefore tried the effect of culturing *Nitrobacter* in vessels fitted with dialysis tubing through which water could be passed from time to time in order to dialyse away accumulated nitrate. Cultures that have oxidized more than 6000 μg. nitrite-nitrogen/ml. are now growing under these conditions with no indication of the onset of any stationary phase in nitrite oxidation or cell growth.

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The Preparation of [6:7-$^3H_2$]Oestrogens. By V. J. O'DONNELL and W. H. PEARLMAN.* (*Royal Infirmary, Glasgow, C. 4, and Guy's Hospital Medical School, London, S.E. 1*)

It is apparent that radioactive oestrogens of high specific activity would be useful in studies of the metabolism of these hormones. Accordingly, we have prepared tritium-labelled oestrogens and have employed 3-acetoxy-oestra-1:3:5(10):6-tetraen-17-one (I), commonly referred to as Δ^6-dehydro-oestrone acetate (Pearlman & Wintersteiner, 1940), as the starting material.

31·8 mg. of I was reduced, with a 10% Pd-charcoal catalyst, in the presence of 100 mc (nominal) of $^3H_2$ to [6:7-$^3H_2$]oestrone acetate (II), m.p. 125·5–126·5°; specific activity 1·04 mc/mg. or 324·5 mc/m-mole (radiochemical yield, 34%). Hydrolysis with methanolic KOH yielded [6,7-^{3}H]-oestrone (III), m.p. 249–251°. There was no diminution in specific activity of the oestrogen molecule. The series of reactions was repeated, starting with 26·8 mg. of I and 3c (nominal) of $^3H_2$. 27·2 mg. of II, specific activity 17·5 mc/mg. or 5·46c/m-mole, was obtained (radiochemical yield, 16%). Alkaline hydrolysis gave III, m.p. 244–247°; specific activity 20·2 mc/mg. or 5·45c/m-mole.

Reduction with sodium borohydride of 32·6 mg. of III, specific activity 0·565 mc/mg. or 152·6 mc/m-mole, afforded [6,7-^{3}H]oestradiol-17β (IV) (26·9 mg.), m.p. 176–178°; specific activity 0·555 mc/mg. or 150·9 mc/m-mole. The yield was 82%.

[6:7-$^3H_2$]Oestriol (V) was prepared following procedures described by Leeds, Fukushima & Gallagher (1954) and by Johnson & Johns (1957). 159·6 mg. of III, specific activity 0·578 mc/mg. or 156·2 mc/m-mole, was treated with *iso*propenyl acetate and toluene-*p*-sulphonic acid to yield [6:7-$^3H_2$]-oestra-1:3:5(10):16-tetraene-3:17β-diol diacetate (VI) (134·5 mg.; 65% yield), m.p. 149–151°; specific activity 0·429 mc/mg. or 151·9 mc/m-mole. Oxidation with perbenzoic acid of 133·2 mg. of VI gave 16α:17α-epoxy-[6:7-$^3H_2$]oestra-1:3:5(10)-triene-3:17β-diol diacetate (VII) (92·6 mg.; 66% yield), m.p. 145–147°; specific activity 0·442 mc/mg. or 163·5 mc/m-mole. 82·4 mg. of VII were reduced with lithium aluminium hydride to give a mixture (mainly V) or 3:16:17-oestriols (64·0 mg.; 100% yield), specific activity 0·474 mc/mg. or 136·7 mc/m-mole.

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The tritium-labelled steroid samples were assayed for radioactivity in a windowless counter according to a method devised by Dr J. F. Tait and co-workers.

We wish to thank Dr C. L. Hewett (Organon Laboratories Ltd.) and Dr A. E. Kellie (Courtauld Institute) for gifts of steroids.

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Foetal and Gastro-Intestinal Glucuronide Synthesis. By G. J. Dutton. (*Department of Biochemistry, Queen's College, Dundee* (*University of St Andrews*))

Glucuronides are synthesized in liver by enzymic transfer of glucuronic acid from uridine diphosphate glucuronic acid (UDPGA) to acceptors (Dutton & Storey, 1954; Storey & Dutton, 1955; Dutton, 1956). In foetal rabbit or newborn mouse-liver slices, *o*-aminophenyl glucuronide (APG) synthesis is deficient (Karunairatnam, Kerr & Levvy, 1949; Hartiala & Pulkkinen, 1955). This is not due to inhibition or glucuronide hydrolysis, but to real deficiencies of enzyme and nucleotide (Dutton & Greig, 1957).

Further work has confirmed absence of 'glucuronyl transferase' and low UDPGA levels in early guinea-pig foetal liver homogenates. Both increased from about half-term, but at birth were still below adult level. Relative synthesis in UDPGA-fortified homogenates was rather higher than in slices, suggesting that lack of available UDPGA contributed to synthetic deficiency, though secondary to lack of enzyme.

With added glucose or glucose 1-phosphate, adult kidney slices were as active as adult liver, but late foetal kidney slices, though more active than foetal liver, were still much less active than adult tissue.

Similarly, in two fresh 3- to 4-month human foetuses liver 'glucuronyl transferase' was negligible, UDPGA low, and kidney slices had only trace activity.

Significant glucuronide synthesis occurs in adult stomach and intestine (Zini, 1952; Shirai & Ohkubo, 1954; Hartiala, 1955). With glucose present, adult guinea-pig stomach and, to a lesser extent, gut, formed a conjugate, identified as APG, at rates up to 30% that of liver (dry-weight basis). Boiled stomach extracts gave evidence of UDPGA, and stomach homogenates, UDPGA-fortified, formed some APG.

With glucose provided, stomach segments were the most active early foetal guinea-pig tissues examined, possessing greater synthesizing capacity than foetal liver or kidney slices, and comparing with adult stomach segments. Intestine appeared less active, and brain, lung and spleen slices inactive.

Even including gastro-intestinal synthesis, foetuses form less APG than adults, probably from deficient 'glucuronyl transferase'. Lathe & Walker (1957) report deficient bilirubin glucuronide formation in newborn jaundiced infants. This may be persisting foetal metabolism, if 'glucuronyl transferase' were involved; which is possible, since deficient formation of phenyl glucuronides occurs in congenitally jaundiced rats and humans (Axelrod, Schmid & Hammaker, 1957; Carbone & Grodsky, 1957).

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The Intracellular Redistribution of Catalase During Mouse Liver Slice Incubation. By D. H. Adams and E. Ann Burgess. (*Cancer Research Department, London Hospital Medical College, London, E.* 1)

Adams & Berry (1956) found that the catalase activity of mouse-liver slices rose when they were incubated in phosphate saline at 38°. During 2 hr. incubation the catalase level approximately doubled, and then remained constant. However, Adams & Burgess (1957) showed that the homogenization technique used by the previous authors, although disrupting the liver cells, left the large granules virtually intact. They also showed that about 75% of the total liver catalase activity was present in the large granule fraction, while the remainder, which was found in solution as soon as the liver cells were broken, appeared to be present in the extra-particulate cytoplasm (EPC). The granule catalase could be liberated by prolonged homogenization or by adding Triton X100, but was not measurable while the granules remained intact.

Adams & Berry (1956) suggested the dissociation of an inactive intracellular catalase complex as a possible explanation of the appearance of the additional catalase activity observed by them. However, subsequent work here has provided no evidence for the existence of such a complex. It was then decided to examine the possibility that the additional catalase measured by Adams & Berry (1956) came from the granule fraction. Measurements were made at intervals during slice incubation of EPC fraction, granule fraction and total catalase. The results showed that the total remained constant and that there was a rise in the EPC fraction which was balanced by a fall in the granule fraction. No evidence has been found that the transfer of catalase from the large granule fraction to the EPC fraction is due either to granule disruption or to an increase in granule fragility during incubation.

When granule preparations were incubated in phosphate saline for 4 hr. no appreciable loss of catalase occurred. However when granules were prepared and incubated in 0·25M-sucrose, catalase was lost from them, the same quantity of enzyme appearing in the supernatant fluid.

It is concluded that during slice-incubation catalase is redistributed in the cell—possibly by migration through the membranes of the large granules into the extra-particulate cytoplasm.

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Some Studies with [1-^{14}C]Succinic Acid. By T. M. Sutherland. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

Topper & Stetten (1954) showed that rabbit-liver slices incubated with [1-^{14}C]succinate in the presence of sulphanilamide gave radioactive acetate in the form of acetylsulphanilamide. In similar experiments with liver slices from rabbit, pigeon and guinea pig we obtained radioactivity in the steam-volatile acids from hydrolysis of the acyl sulphanilamide fraction. Chromatography of the steam-volatile acid (Bueding & Yale, 1951) indicated that radioactivity was due to two components one of which was acetate. Chromatography (Pennington & Sutherland, 1956) of the [1-^{14}C]succinic acid (Radiochemical Centre, Amersham, Bucks) revealed the presence of a steam-volatile acid accounting for 0·2% of the total radioactivity. This impurity was identified as propionic acid. Using [1-^{14}C]succinic acid purified by permanganate treatment and chromatography, the radioactivity isolated as acetate and propionate after incubation experiments with liver slices from rabbit, pigeon and guinea pig was less than 0·002% of the total radioactivity of succinate added; a radiochemical purity better than 99·998% would therefore be required to give significance to the formation of labelled acetate from [1-^{14}C]succinate. The proportion of acetate coming from cleavage of succinate in these experiments, assuming no interference by radiochemical contamination, was 0·05% or less.

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The Effect of Reserpine on the Blood Sugar Level in Human Subjects. By ELIZABETH F. MARSHALL, A. C. TAIT and A. TODRICK. (*Department of Clinical Research, Crichton Royal, Dumfries*)

Reserpine, the major alkaloid of *Rauwolfia serpentina*, has been employed therapeutically in the treatment of hypertension since 1951 and more recently as a tranquillizing drug in the treatment of psychotic types of mental illness. It has been shown to possess a specific effect on the metabolism of 5-hydroxytryptamine (5-HT), which is mobilized from the body depots and excreted in the form of 5-hydroxyindolylacetic acid (5-HIAA). Earlier observations on animals, using high doses of the alkaloid, have recently been confirmed in tests on human subjects receiving therapeutic doses (Haverbach, Shore, Tomich & Brodie, 1956; Todrick, Dick & Tait, 1958).

It had been postulated that reserpine exerted its pharmacological action by inactivating the 5-HT-binding sites of the depots, but it has recently been shown that it modifies the body content of adrenaline to a similar degree. In attempting to distinguish between possible alternative modes of action of reserpine, the significance of a blood sugar rise as an indicator of adrenaline action has been considered. Earlier reports relating to the effect of reserpine on blood sugar levels reach contradictory conclusions (Kuschke & Frantz, 1955; Neugebauer & Lang, 1953; Schuler, 1955).

Tests have been carried out on ten patients receiving an intramuscular injection of 5 mg. reserpine; these included blood pressure, blood sugar and urinary 5-HIAA estimations. In two cases blood pressure and 5-HIAA excretion remained stationary, but in the remainder the maximum observed fall in systolic blood pressure averaged 28 %, while the increase in 5-HIAA excretion in the four hours following the injection averaged 80 %. Changes in blood sugar at 30 and 90 min. after the injection were slight and irregular, though there was a progressive fall in the mean level of the group, significant only at 90 min.

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Fatty Acids in the Liver Lipids from Olive Oil-fed and Cholesterol-fed Rats. By J. B. M. RATTRAY,* A. T. JAMES and R. P. COOK. (*Biochemistry Department, Queen's College, Dundee (University of St Andrews) and National Institute for Medical Research, Mill Hill, London, N.W. 7*)

Rats were fed diets containing 16 % olive oil without (normals) and with 1·6 % added cholesterol (sterol-fed). The liver lipids (ether and ethanol extracts) were separated by acetone at various temperatures into primary fractions. These were chromatographed on silicic acid-Celite; the subfraction saponified, unsaponifiable matter removed, the fatty acids recovered, converted to methyl esters and their composition determined by gas-liquid chromatography (James & Martin, 1956). The fractions were analysed for free and total sterol and for glycerol.

For normal liver the acids accounted for 58·1 % of the lipid extract and had the following distribution (as % of total fatty acid recovered), and composition: one sterol ester fraction (3·4 %) mainly oleic and palmitic acids; two mixed ester and glyceride fractions (8·6 %) containing oleic acid, C_{20} polyunsaturated acids; palmitic acid and a C_{12} branched or unsaturated acid; a main glyceride fraction (48·3 %) with oleic and palmitic acids predominating; two more polar glyceride fractions (9·7 %) containing palmitic, oleic and stearic acids; the 'phospholipid' fraction (29·8 %) composed of oleic, palmitic and stearic acids with di- and poly-unsaturated acids.

The liver lipid extract of sterol-fed animals had a fatty acid content of 40 %, distributed as three sterol ester fractions (35·8 %), consisting mainly of linoleic and oleic acids; a fraction (3·3 %) containing volatile unsaponifiable matter, the acids consisting mainly of oleic and palmitic acids and a C_{12} branched or unsaturated acid; four less polar glyceride fractions (36·4 %) mainly of oleic and palmitic acids, but of one rich in the branched C_{12} acid; four more polar glycerides (22·9 %), one rich in a highly branched C_{12} acid; the 'phospholipid' (1·6 %) containing acids similar to those in the normal livers.

A great variety of odd and even numbered, saturated and unsaturated, straight and branched-chain fatty acids were demonstrated.

This work was aided by an expenses grant from the Scottish Hospitals Endowments Research Trust.

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Ketonic Products of Deoxycorticosterone Metabolism by Rabbit-Liver Homogenate. By W. TAYLOR. (*Department of Physiology, Medical School, King's College, Newcastle upon Tyne* 1)

In a previous communication (Taylor, 1957) the isolation of 3β:21-dihydroxy-5α-pregnan-20-one, 3α:21-dihydroxy-5α-pregnan-20-one and of unchanged deoxycorticosterone (DOC) after incubation of the latter with rabbit-liver homogenate was reported. Further analysis of the α (not precipitated by digitonin)-ketonic fraction has revealed the presence of the following metabolites: 20α:21-dihydroxypregn-4-en-3-one and its 20β epimer (I), with the former predominating, 3α:21-dihydroxy-5β-pregnan-20-one and 21-hydroxy-5-pregnane-3:20-dione. Identifications were based on paper chromatography in at least two solvent systems of the free and acetylated steroids and on colour reactions with alkaline blue tetrazolium (BTZ), u.v. light, 15% and 75% H_3PO_4, conc. H_2SO_4 and HI/I_2. The identity of I was further established by infra-red spectroscopy.

Four other unidentified substances (II–V) have also been isolated in small quantities. All reduced BTZ, and their paper chromatographic properties indicated that the substances contained an extra oxygen function. II also absorbed u.v. light: the free steroid had the same R_F as that of 14α-hydroxy-DOC, but after treatment with excess acetic anhydride in pyridine the product had the same R_F as 6β:21-diacetoxypregn-4-ene-3:20-dione. III–V did not absorb u.v. light and were more 'polar' than hydroxylated derivatives of DOC with the hydroxyl group in an equatorial conformation. These findings suggest that III–V are derivatives of DOC reduced in ring A and possessing more than three oxygen atoms per molecule. The possible identity of these substances will be discussed.

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Metabolic Studies using Aseptically Cultured *Drosophila melanogaster*. By J. H. SANG (introduced by D. J. BELL). (*A.R.C. Poultry Research Centre, West Mains Road, Edinburgh* 9)

Studies of intermediary metabolism have been facilitated by the use of micro-organisms with particular genetic defects (metabolic 'blocks'). Little has been done yet to use higher organisms in a similar fashion, although such studies should extend the metabolic picture into the areas of development and morphogenesis. The reasons for this will be discussed briefly and some preliminary work on the definition of the normal metabolism of the genetecist's organism of choice (*Drosophila melanogaster*) will be described.

As the simplest approach to metabolism, in this context, is by way of nutritional studies under aseptic conditions, the primary problem is the definition of an optimal diet. Departures from this optimum can thus be used to explore metabolism at the general level and, as an example, the effects of high and low protein supplies on vitamin requirements will be described. Two cases where high protein involves an increased vitamin requirement will be analysed in some detail: biotin and folic acid. Since the latter substance appears to be necessary to *Drosophila* for the same three functions commonly found with vertebrates (purine synthesis, serine–glycine interconversion and methylation of 2-deoxyuridine to thymidine) the effects of specific inhibition of the last of these functions during development will be described as an instance of interference at the morphogenetic level. Non-hereditary changes, identical with those caused by known genes, can be produced in this way.

Levels of Two Erythrocyte 'Non-Protein Nitrogen' (NPN) Fractions of Hens in Different Physiological States. By D. J. BELL. (*A.R.C. Poultry Research Centre, West Mains Road, Edinburgh* 9)

Observations of the NPN of blood filtrates using trichloroacetic acid (Bell, 1957*a*) have been extended to the ninhydrin-N ('NN') (Yemm & Cocking, 1955) and the NPN minus NN, or 'rest-N' ('RN') levels of plasma and erythrocytes in three physiological states. Groups of 12 fed birds gave the following mean values:

Plasma-N (mg./100 ml.). Layers: NN, 7·8 (range 5·4–11·6, S.D. 1·6); RN, 7·5 (range 2·8–13·4, S.D. 3·0). Non-layers: NN, 8·3 (range 4·6–11·5, S.D. 1·8); RN, 11·2 (range 8·7–15·7, S.D. 2·2). Immatures (6 weeks old): NN, 9·1 (range 4·0–14·3, S.D. 2·9); RN, 8·2 (range 4·0–15·0, S.D. 3·8). These plasma amino nitrogen levels are similar to the amino acid

levels found by Bock (1917) and Salander & Fisher using respectively the Van Slyke and Danielson methods.

Erythrocyte-N (mg./100 ml.). Layers: NN, 31·9 (range 23·0–40·5, S.D. 5·3); RN, 79·3 (range 66–104, S.D. 12·3). Non-layers: NN, 43·0 (range 29·8–61·0, S.D. 9·3); RN, 62·4 (range 31–80, S.D. 15·2). Immatures: NN, 50·5 (range 40·0–64·0, S.D. 7·5); RN, 93·4 (range 80–116, S.D. 11·4).

Statistically significant differences are as follows: *Plasma*: RN, layers < non-layers ($P < 0{\cdot}01 > 0{\cdot}001$). *Erythrocytes*: NN, layers < non-layers ($P < 0{\cdot}05 > 0{\cdot}02$); RN, layers > non-layers ($P < 0{\cdot}02 > 0{\cdot}01$). Comparing immatures with non-laying adults both NN and RN levels in the corpuscles are obviously higher in the rapidly growing birds.

The source of about 40–70 mg. of erythrocytes RN is unknown; uric acid is absent (Benedict, 1915; McIndoe, unpublished). From the literature about 22 mg. of RN can be assessed from known sources as follows (in the fowl urea (Bell, unpublished) and creatine (Wilson & Plass, 1917) are approximately equally distributed between cells and plasma); urea, 0·5–1·5 (Bell, 1957*b*); creatine, ~0·3 (Salander & Fisher, 1956); ATP, 7–9 (Rapoport & Guest, 1941); ergothioneine, up to 4 (Hunter, 1928); glutathione, 7 (Chanton, 1932).

It is suggested that nitrogenous small molecules can be carried by the erythrocytes to supply extravascular metabolic demands.

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Total Sulphydryl (SH) Content of Blood and Tissues. By S. K. Bhattacharya (introduced by D. G. Wild). (*Department of Biochemistry, University of Leeds*)

Recently Benesch, Lardy & Benesch (1955) have published an improved amperometric method for the determination of SH groups in simple thiols and in proteins. The method has three distinct advantages over other commonly used methods, namely, proteins are not denatured in the course of the determination; the titratable free SH groups of protein and those of non-protein substances react with silver nitrate under the same experimental conditions; and the colour and turbidity of the specimens to be analysed do not interfere in the procedure. The present work was undertaken to examine the applicability of this method to the determination of total SH groups (non-protein SH + free SH groups of protein) in blood and in tissue homogenates.

Rat blood (obtained from the abdominal aorta) and tissues were used in this work. 0·1 ml. of whole blood haemolysed in 1 ml. of deionized distilled water and 1–2 ml. of 10% (w/v) tissue homogenates were titrated with 0·002N silver nitrate according to the method of Benesch *et al.* In a few preliminary experiments, samples of the same tissue were homogenized in three media, namely, deionized distilled water, 0·25M-sucrose solution, and 0·15 M-KCl + 0·005 M-$MgCl_2$ + 0·005 M-ethylenediaminetetra-acetic acid (disodium salt) solution, pH 7·0, and the SH content determined. The SH content of the sucrose homogenate was found to be lower than those of the water and KCl homogenates. In later work, SH determinations were done only in water homogenates. The results of several experiments indicated that the method of Benesch *et al.* was not specific for the determination of SH groups of protein in tissue homogenate and therefore the method was modified. The modification involved a blank titration after blocking the SH groups with *p*-chloromercuribenzoic acid. The total SH content of whole blood, liver and pancreas was determined by the modified technique and the following mean values were obtained: Blood, 91·4 mg./100 ml. (range 83·6–100·0; 8 determinations); liver, 55·4 mg./100 g. (range 51·4–58·8; 7 determinations); and pancreas, 22·3 mg./100 g. (range 20·0–25·0; 3 determinations).

The author wishes to thank Professor F. C. Happold for giving the necessary laboratory facilities, and the University of Leeds for an I.C.I. Fellowship which made this work possible.

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DEMONSTRATION

An Apparatus for Zone Electrophoresis in Vertical Packed Columns. By D. L. MOULD* and J. STEPHEN. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

Zone electrophoresis in packed columns is now a standard technique in biochemical research and many types of basically similar apparatus have been constructed. It is essential to maintain a wide cross-section throughout the connexions between electrode vessels and column and minimize ohmic losses in field strength. The apparatus demonstrated is a simplified version of the design of Porath (1956). The column 3·0 × 60·0 cm. has a sintered disk sealed into a B34 cone at the bottom end and is inserted into the centre conical joint of a three-necked flask (5 l.) used as anode vessel. A glass bend (5 cm. diam.) with filling taps connects the top of the column through a B34 conical joint to a similar cathode vessel placed approximately at the same level. Sleeved platinum electrodes are inserted in the side sockets of the flasks. Electrolytic gases escape through vertical tubes above the electrodes. Narrow vertical tubes are placed in the other flask sockets. During electrophoresis electro-endosmotic flow causes a rise in the hydrostatic head in the cathode vessel until equilibrium is established. During prolonged electrophoresis with solutions of low buffering power the anode vessel is perfused from a constant-head device, the flow being directed upwards through the sleeved electrode. The column is removed for elution by lowering the anode vessel. The effective field across the column is about 80% of the applied p.d. Potato starch or ethanol-treated cellulose (Flodin & Kupke, 1956) packed columns have been successfully employed for some time in this apparatus for the fractionation of animal sera, enzyme and toxin preparations under varied buffer conditions.

A design of zone electrophoresis apparatus based on that described is available from Quickfit and Quartz Ltd., Stone, Staffs.

We wish to thank Mr H. Harris, Dept. of Chemistry, University of Aberdeen for construction of the apparatus.

* Now at the Animal Diseases Research Association, Moredun Institute, Edinburgh.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **374th Meeting** *of the Society was held in the Department of Biochemistry, Tennis Court Road, Cambridge, on Tuesday, 24 June and Wednesday, 25 June* 1958, *when the following papers were read:*

COMMUNICATIONS

The Effect of Chemical Modification on the Uptake of Cobalt (II) Ions by Human Serum Albumin, in Aqueous Solution. By A. CODDINGTON and D. J. PERKINS. (*Department of Chemical Pathology, St George's Hospital Medical School, London, S.W.* 1)

The binding of native and chemically modified human serum albumin with bivalent cobalt ions has been studied, in an attempt to elucidate both the extent of binding, and the nature of the binding sites. Equilibrium dialysis experiments were carried out in the pH range 4–8, using solutions containing 5 g./l. of protein, and 10 μg./ml. of cobalt. The difficult analytical technique for the measurement of microquantities of cobalt was obviated by using ^{60}Co as an isotopic tracer. The polar side-chains of human serum albumin, in particular the ϵ-amino groups of lysine, and the free carboxyl groups of glutamic and aspartic acids, were chemically modified by standard techniques. Chemical and physical methods were used to determine the number of groups modified, and the extent of denaturation of the protein. The blocking of the ϵ-amino groups by acetylation, deamination, succinylation, and reaction with 2:4-dinitrobenzenesulphonic acid, caused a significant increase in the binding, over the native protein. The blocking of the free carboxyl groups, by esterification with methanol, and with propylene oxide, caused a small decrease in binding; this decrease being especially marked at high pH values. Preliminary considerations lead to the conclusion that the binding of cobalt to serum albumin is probably non-specific in character, though there is evidence that the imidazole residue may be responsible at pH values up to 6·5.

The Crystalline Style of *Pinna nobilis*. By KENNETH BAILEY. (*Department of Biochemistry, University of Cambridge and Stazione Zoologica, Naples*)

In bivalves, the crystalline style exists as a gelatinous rod lying in a coecal pouch of the stomach. Its rotation by cilia attached to the border cells causes the head of the style to disperse by friction against the gastric shield, whilst the body of the style is simultaneously renewed by special secretory cells. The dissolution of the matrix of the style causes the liberation of an amylase (Coupin, 1900), but the amount of the latter is small compared with the matrix itself, about which little detailed chemistry is known.

During an extended investigation of the tropomyosin (paramyosin) component of *Pinna* adductor muscle (Bailey, 1956, 1957), the styles of all animals used were isolated and solubilized at pH 8. A somewhat empirical fractionation procedure (carried out at Naples) yielded two fractions, one precipitating at pH 5 (A) and the other (B) from the mother liquor by acetone. By suitable chromatographic methods, both were found to consist of a protein-carbohydrate complex giving rise on hydrolysis to amino acids, hexosamine, galactose and fucose, the hexosamine consisting of equal parts of glucosamine and galactosamine. The amounts present as percentage of the dry weight in the case of fraction A were: hexosamine 12·4, hexose, 11·5, methylpentose 6·5. Fraction B was analytically similar. Tests for uronic and sialic acids were negative. In the ultracentrifuge, fraction A resolved itself into two quite symmetrical peaks (S_{20} 5·0 and 8·4 for 0·9 % solution) with no trace of minor components.

The character and relative amounts of the carbohydrate components are so like that of a blood group substance (especially A substance) that Professor W. T. J. Morgan kindly consented to test Fraction A for A, B, H and Le^a activity. The preparation was entirely inactive.

It is concluded that the crystalline style, apart from trace enzyme components, consists of mucopolysaccharide complexes of which about 70 % is protein and the rest glucosamine, galactosamine, galactose and fucose.

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The Possible Function of Invertebrate Tropomyosin. By J. C. Rüegg. (*Department of Biochemistry, University of Cambridge*)

Invertebrate tropomyosin (Bailey, 1956) which is so plentiful in certain lamellibranch adductor muscles does not seem to take an active part in contraction, since all the ATP-ase activity of the muscle is associated with a separate actomyosin-like protein (Rüegg, 1957). Its abundance in definite fibrils (Hanson, Lowy, Huxley, Bailey, Kay & Rüegg, 1957) indicates some important mechanical or structural role. Two recent findings suggest that it may be connected with the viscous tonus of such muscles and that interactions with ATP or with Ca ions may account for changes in 'muscle viscosity' of the type described by Winton (1937). First, the smooth adductor of *Pecten* is now found to consist of two distinct parts, that nearest to the striated adductor having an ATP-ase activity 5 times higher than the more opaque external part. By electrophoretic and ultracentrifugal analysis only 15–25% of the total globulin in the latter consists of actomyosin, the rest being tropomyosin. In the other portion the amounts of actomyosin and tropomyosin are about equal. Preliminary experiments, carried out with Dr W. Hasselbach, showed that for glycerated fibres contracted isometrically the rate of stress-relaxation and of the recovery after quick release is much slower in the tropomyosin-rich fibres, whilst the resistance to stretch is higher. Secondly, the colloidal state of invertebrate tropomyosin is affected by ATP and by Ca ions. For example, at low ionic strength its solubility is greatly increased by 0·005 M-ATP, which becomes adsorbed. ATP also increases considerably the electrophoretic mobility. Artificial threads made by the Hayashi method become more extensible in presence of ATP and less so in presence of Ca ions, an effect also shown by fibre bundles after selective denaturation of actomyosin. Presumably the plasticizing action of ATP on tropomyosin systems is due to a decrease of protein-protein interaction caused by binding of ATP.

I am greatly indebted to Professor H. H. Weber for his hospitality during part of this work.

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The Extent of the Incorporation of Different Amino Acids into Proteins of Cell-free Systems of Rat Spleen and Liver. By R. Rendi* and P. N. Campbell. (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1*)

The incorporation of different amino acids into the proteins of cell-free preparations of liver has been extensively studied, but there have been few such reports concerning other mammalian tissues. Wagle, Mehta & Johnson (1958) studied the supernatant obtained from a homogenate of rat spleen which had been centrifuged at 8000 *g*. When this preparation, which probably contains mitochondria, was incubated with fructose-diphosphate as energy donor, alanine, methionine and phenylalanine were incorporated to the same extent as in a similar preparation from rat liver.

These experiments of Wagle *et al.* (1958) have been repeated, with minor modifications of the experimental conditions, and the incorporation of various amino acids (glycine, alanine, leucine, glutamic acid and lysine) has been studied. While in some cases the extent of incorporation in liver and spleen was similar, e.g. alanine (71 counts/min. for liver and 68 for spleen), in other cases, for example, leucine, the incorporation was much greater in liver than spleen (381 and 82 counts/min. respectively).

In order to determine whether these differences in the extent of amino acid incorporation were associated with the microsomes, or were due to the presence of mitochondria, a microsome-cell sap fraction, freed from mitochondria by centrifugation at 12 000 *g*, was incubated anaerobically with phosphoenolpyruvate (PEP) as energy source. In this system, also, alanine was incorporated to the same extent in the two tissues while leucine was much more actively incorporated into the liver than into the spleen preparation. An attempt was then made to study the incorporation in isolated microsomes. Since the microsomal fraction from rat spleen, obtained by centrifugation at 105 000 *g*, had only a low activity, the microsomal fraction obtained by precipitation with $MgCl_2$ (Siekevitz, 1952) was studied. When such a preparation was incubated with 'pH 5 enzymes', PEP, guanosine triphosphate, glutathione, adenosine triphosphate

* British Council Scholar from Italy.

and phosphoenolypyruvate kinase, an energy-dependent incorporation of radioactive amino acids was obtained. In such a system the incorporation of alanine and leucine was similar (46 and 56 counts/min. respectively). With a preparation of liver microsomes, obtained by centrifugation at 105 000 *g*, leucine was much more actively incorporated than alanine.

These experiments suggest that differences found in the extent of incorporation of alanine and leucine in cell-free preparations of rat liver and spleen are a characteristic of the microsome-cell sap fraction of these tissues.

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Amino Acid 'Exchange' and Protein Synthesis in Cell Walls of *Bacillus megaterium*. By A. R. Crathorn and G. D. Hunter. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W.* 3)

It has been shown by Gale & Folkes (1953) that single radioactive amino acids may still be incorporated into the 'protein' (material insoluble in hot trichloroacetic acid) of *Staphylococcus aureus* even when a net increase in protein is prevented by the presence of chloramphenicol. More recently, Mandelstam & Rogers (1958) and Hancock & Park (1958) have shown that much of the incorporation of labelled amino acid under these conditions of amino acid 'exchange' represents synthesis of the hexosamine-peptide polymer which is a major constituent of the cell wall.

However, the methods employed by both these pairs of workers do not distinguish between the labelling of the protoplast membrane and cytoplasm, nor, with certainty, between the cell wall and the protoplast membrane. In the case of *Bacillus megaterium*, where we have observed an 'exchange' reaction analogous to that observed in the *Staphylococcus*, these three cellular fractions may be clearly separated. It has been found that in the presence of chloramphenicol a [^{14}C]amino acid (L-alanine), that occurs in the cell wall of *B. megaterium*, is incorporated only to a very slight extent into the cytoplasmic proteins, but appears in the proteins of both the cell wall and the protoplast membrane. The proportion of the label present in the cell wall and protoplast membrane varies with time, and it appears likely that, as in the case of the cytoplasmic protein (Butler, Crathorn & Hunter, 1958), the cell-wall protein is first elaborated in the protoplast membrane.

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Presumed Nuclear Bodies Isolated from Yeast Protoplasts using Ultrasonics. By A. A. Eddy. (*Brewing Industry Research Foundation, Nutfield, Surrey*)

Most work with isolated nuclei has hitherto been carried out with preparations from animal tissues rather than from micro-organisms such as yeast, the latter indeed presenting a special problem in not possessing a nucleus which has been incontrovertibly identified. In the present study a method has been devised whereby the deoxyribonucleic acid (DNA) contained in the yeast protoplast (Eddy & Williamson, 1957) can be isolated in association with a body of visible dimensions.

To prepare such material, chilled portions (20 ml.) of a suspension of protoplasts (4×10^8/ml.) in 0·01 M-acetate buffer at pH 5 containing 1 mM-$MgCl_2$, 0·1 mM-$CaCl_2$ and 0·56 M-mannitol were exposed for 40 sec. to ultrasonic vibrations (650 kcyc./sec.) damped by transmission through an oil-bath. A fraction sedimenting at 1000 *g* was separated, washed twice with a solution of the above composition, freed from coarse debris and most residual protoplasts by fractional centrifuging at 10 *g* and then washed three times with a similar medium but lacking mannitol.

The material so obtained, representing about 20% of the nitrogen content of the protoplast, consisted largely of individual bodies of about 2 μ diameter which, however, tended to aggregate. Analysis gave: DNA, 1%; RNA, 15% (w/w), these being extracted using 0·5 N-perchloric acid at 70° for 20 min. and assayed by the methods of Burton (1956) and Mejbaum (1939) respectively.

As would be expected, the DNA was found to be removed by deoxyribonuclease but not by ribonuclease.

If the sedimentable DNA were merely adsorbed accidentally to the bulk of the preparation, addition of thymus DNA prior to treatment of the protoplasts with ultrasonics might have increased the amount of DNA sedimenting. No such effect occurred, however, so this explanation can probably be rejected.

Using a special technique in which protoplasts were transferred to formamide and also disrupted in this medium, evidence was obtained suggesting that each protoplast contained a single body of the above kind situated just outside the main vacuole.

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The Induced Formation of an Amino Acid Decarboxylase. By BARBARA G. HAUGHTON and H. K. KING. (*Department of Biochemistry, University of Liverpool*)

Proteus vulgaris produces an enzyme which can decarboxylate L-leucine, L-valine, L-isoleucine and α-amino-*n*-butyric acid (Ekladius, King & Sutton, 1957). Activity is low in washed suspensions prepared from cells grown on a medium containing no amino acids but increases when the cells are incubated with leucine or valine and a metabolizable substrate such as lactate. The rate of enzyme formation is greater, however, if the inducer is added to a growing culture; increase in enzyme activity is then proportional to increase in culture density.

Several compounds have been tested for their ability (*a*) to combine with the enzyme as substrate or inhibitor; and (*b*) to induce formation of the enzyme in growing cultures. All known substrates of the enzyme act as inducers, whilst D-valine, L-lysine, DL-γ-amino-*n*-butyric acid and DL-amino*iso*butyric acid are neither substrates, inhibitors, nor inducers. DL-Alanine induces enzyme formation but is neither a substrate nor an inhibitor. *iso*Butylamine and *iso*valerate inhibit the enzyme but are not inducers. *N*-Acetyl-L-leucine inhibits the enzyme but induces its formation, possibly after hydrolysis to leucine. Phenylalanine, neither a substrate nor an inhibitor of enzyme action, inhibits the leucine-induced enzyme formation.

The Michaelis constant (K_s) of the cell-free enzyme is about 0·01 M for leucine, valine and isoleucine; valine gives a slightly higher value in intact cells. Half-maximal induction is obtained, however, at 0·001 M with leucine, valine, or isoleucine.

These results support the view that inducers act through combination with a receptor distinct from the enzyme itself, and showing, in some cases, different specificity.

We are grateful to the Medical Research Council for a grant towards expenses and for a scholarship for one of us (B.G.H.).

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Preparative Immunoelectrophoresis. By T. H. MEAD. (*The C.S.I.R. and University of Cape Town Virus Research Unit, Medical School, Observatory, Cape, South Africa*)

This method of removing antigenic impurities from biological extracts depends on specific precipitation and can only be used when an antiserum is available which contains antibodies capable of precipitating the impurities but not the required constituents of the extract. These constituents must also migrate anodically on electrophoresis in agar at pH 7·6.

The optimal-proportions condition for complete precipitation is difficult to satisfy when two or more antigen-antibody systems are present. Moreover, the usual method contaminates the extract to be purified with residual constituents of the antiserum. The new method depends on the conditions present in the Oakley & Fulthorpe (1953) modification of the Oudin gel-precipitin technique and consists in causing the constituents of the extract to migrate in agar in an electric field through a concentration gradient of antibodies moving (owing to electroendosmosis) in the opposite direction. Each unwanted antigen thus meets a gradually increasing concentration of its antibody until the proportions are optimal and a stationary band of precipitate is formed. The remaining (negatively

charged) constituents of the extract pass through the gradient, emerge from the agar column and are collected. Preliminary electrophoretic purification of the γ-globulins ensures that no constituents of the antiserum contaminate the product.

The apparatus consists essentially of a U tube in one arm of which a column of agar can be built up while the U is inverted. The remainder of the tube including a space above the agar is filled with buffer and the top of each limb connected to a non-polarizable electrode. On the side containing the agar a cellophan membrane prevents proteins which migrate into the supernatant buffer from reaching the electrode.

The method was designed to purify the soluble antigen of rabies virus but may be useful in other fields. As an illustration the purification of serum albumin will be discussed.

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A New Estimation of Ergothioneine and its Association with Blood Proteins. By P. C. JOCELYN. (*Department of Clinical Chemistry, University of Edinburgh*)

The estimation of ergothioneine by the colorimetric method of Hunter (1928) is not satisfactory even after modification (Lawson, Morley & Woolf, 1950; Hunter, 1949) due to the presence of other substances in blood and tissues which themselves react with the diazotized sulphanilic acid reagent used.

A new method is now described involving the quantitative cleavage of trimethylamine from ergothioneine with 50% alkali at 100° during 1 hr., aeration of this amine from the solution and, after nesslerization, its spectrophotometric determination as the benzene-soluble picrate. There is no interference from added ammonia and primary amines nor from betaine and choline, neither of which release their trimethylamine residues under these conditions.

When ergothioneine is added to human blood, a quantitative recovery is obtained by this method on the solution subsequently deproteinized by heat and acetic acid. Nevertheless, a lower range for the concentration in normal human blood (1·0–2·6 mg.%) and packed cells (1·8–5·0 mg.%) is found than has been reported by users of the Hunter method (Hunter, 1949; Lawson *et al.* 1950; Latner & Mowbray, 1949; Melville & Lubschez, 1953). On the other hand, ergothioneine cannot be estimated in the plasma by the latter method (Hunter, 1949; Fraser, 1950) but is found to be present consistently in small amounts in human plasma by the new technique (average: 0·35 mg./100 ml.). This plasma concentration is not derived by diffusion of ergothioneine out of the blood cells for this does not occur even after prolonged incubation of separated cells with normal saline.

The proteins precipitated by heat and acetic acid also contain ergothioneine as estimated by this method. The amounts from 100 ml. of blood, packed cells or plasma are as follows; plasma proteins: 1·0–1·6 mg.; blood proteins: 2·3–2·9 mg.; packed cell proteins: 2·7–3·4 mg.

The value for a given plasma protein fraction is unaffected by dialysis of the plasma or treatment with reducing agents prior to precipitation of the proteins.

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Lipid Components in Nucleohistone. By J. CHAYEN and P. B. GAHAN. (*Department of Pathology, Royal College of Surgeons, Lincoln's Inn Fields, London, W.C.* 2)

Calf-thymus nucleohistone gives positive histochemical tests for phospholipids (Baker, 1946) and yields a 60 Å spacing similar to that given by sphingomyelin (Wilkins, 1956). If the histochemical results are 'spurious' the detection of lipids in intact chromosomes is impossible; if they are due to phospholipids, the lipids are resistant to solvents. It was considered advisable, therefore, to test these alternatives by examining the hydrolytic products of high-polymer nucleohistone for appreciable quantities of lipid-degradation substances.

The calf-thymus nucleohistone (2·77 g. dry wt.) was prepared by Zubay's (1957) modification of the

method of Shooter, Davison & Butler (1954) and was subjected to the hydrolytic procedure of McKibben & Taylor (1949) for the estimation of sphingosine. On shaking the hydrolysate with $CHCl_3$ to remove any free 'sphingosine' a heavy pale precipitate formed. The aqueous phase was examined by paper chromatography (see Lovern, Olley, Hartree & Mann, 1957) and glycerophosphate and choline (59 mg.) were detected. The nitrogen content (0·19 mg.) of the $CHCl_3$ phase was determined by a micro-Kjeldahl and Nessler technique. The precipitate was refluxed for 5 hr. in methanolic 5N-HCl and then shaken with $CHCl_3$. Nitrogen (2·8 mg.) was found in the $CHCl_3$ and only free H_3PO_4 in the aqueous phase. To determine whether this nitrogen was due to solution of nucleic acid bases or amino acids in the $CHCl_3$, some of this phase was run as a paper chromatogram in *n*-propanol-water (80:20, v/v). Apart from material which corresponded to sphingosine, there was no ninhydrin-positive matter; a small spot, R_F 0·59, $\lambda_{max.}$ in N-HCl = 263 mμ, was detected. If this be calculated as thymine it would have contributed only 0·08 mg. nitrogen to the $CHCl_3$ extract.

Pure nucleohistone should not have yielded any $CHCl_3$-soluble nitrogen. If this nitrogen were present as sphingosine in a sphingomyelin of normal structure, it would comprise some 6% of the nucleohistone.

Hydrolysis of another sample (dry wt. 2·81 g.) in ethanolic KOH and then in 6N-HCl yielded 350 mg. of glycerophosphate and 120 mg. of choline, estimated by paper chromatography.

We are grateful to Dr C. Long, Dr J. A. Lovern and Dr J. Olley for very helpful advice, and Dr G. L. Zubay for help in preparing the samples.

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Plasmalogen in Ram Semen and its Role in Sperm Metabolism. By E. F. HARTREE and T. MANN. (*A.R.C. Unit of Reproductive Physiology and Biochemistry, Molteno Institute and Department of Veterinary Clinical Studies, University of Cambridge*)

For a long time it has been generally accepted that the phospholipid present in mammalian spermatozoa consists chiefly of lecithin. Recently, however, it has been shown that there is little or no lecithin in ram spermatozoa and that the predominant intracellular phospholipid of ram semen is a choline-based plasmalogen (Lovern, Olley, Hartree & Mann, 1957).

The present study has been concerned with the content, properties and metabolic behaviour of the plasmalogen present in ram spermatozoa. If the mean molecular weight of the sperm plasmalogen is assumed to be 685, 100 ml. ram semen contains 0·38 g. plasmalogen of which about 90% is present in the spermatozoa and the remainder in the seminal plasma. The sperm cells contain about 3·8 g. plasmalogen/100 g. dry weight: this concentration is higher than that recorded in most other animal tissues.

When spermatozoa are incubated in the presence of fructose, i.e. as whole semen or as a suspension in a fructose-containing Ringer solution, there is no net breakdown of plasmalogen. However, in the absence of fructose there is a progressive decrease in the acyl ester content of the sperm lipid. A similar but more rapid decrease takes place when the spermatozoa are incubated with snake venom. In both cases the plasmalogen appears to lose one molecule of fatty acid and to yield a lysoplasmalogen. This change, however, is not associated with any appreciable decrease in the intracellular content of either lipid-phosphorus or fatty aldehyde. Measurements of the respiratory quotient of aerobically incubated suspensions of washed ram spermatozoa gave a mean value of 0·71. This figure suggests that the fatty acids liberated from plasmalogens can serve as substrate for the aerobic metabolism of ram spermatozoa suspended in a fructose-free medium.

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The Biosynthesis of Porphyrins from Porphobilinogen by *Rhodopseudomonas spheroides*. By D. S. Hoare and H. Heath. (*Department of Chemical Pathology, University College Hospital Medical School, London, W.C.* 1)

Washed suspensions of *Rhodopseudomonas spheroides* synthesize free porphyrins from glycine together with certain intermediates of the tricarboxylic acid cycle (Lascelles, 1956). Such suspensions were unable to convert added porphobilinogen to porphyrins. It has now been shown that frozen and thawed cell suspensions incubated with porphobilinogen form coproporphyrin III together with traces of uroporphyrin. Bogorad & Granick (1953) made similar observations with *Chlorella*. Working at pH 6·5 with suspensions (4–40 mg. dry weight/ml.) of anaerobic/light grown cells, the formation of coproporphyrin from porphobilinogen over a 4 hr. incubation period was proportional to the amount of cells present.

When cell suspensions were repeatedly frozen and thawed in fresh batches of buffer, or in water, the ability to form coproporphyrin from porphobilinogen was lost, and instead large amounts of uroporphyrin III were formed. The cell-free buffer or water solutions resulting from this process, although containing very low concentrations of protein, were able to convert porphobilinogen to uroporphyrin I. If the system were reconstituted by adding the extracts to the extracted cell suspensions, porphobilinogen was converted to coproporphyrin, as originally observed.

Cell-free extracts, obtained after grinding with alumina, formed both uroporphyrin and coproporphyrin. With high concentrations of extract coproporphyrin exceeded the uroporphyrin formed; with low concentrations far more uroporphyrin than coproporphyrin was formed. The uroporphyrin was a mixture of the I and the III series isomers.

Acetone-dried cells were most active in forming coproporphyrin from porphobilinogen. As with cell-free extracts uroporphyrin was the main reaction product at low enzyme concentrations. In the initial stages of coproporphyrin formation either uroporphyrin or uroporphyrinogen accumulated. The total estimated uroporphyrin, which included uroporphyrinogen, decreased with time as the concentration of coproporphyrin increased. Since added uroporphyrinogen was converted to coproporphyrin whereas neither uroporphyrin I nor III was further metabolized, the apparent high initial uroporphyrin concentration was attributed to the presence of uroporphyrinogen. Confirmation of this was obtained from ultra-violet-absorption measurements. Booij and Rimington (Rimington, 1957) also suggested that uroporphyrinogen might be formed initially in avian haemolysate systems.

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Sulfmyoglobin and Sulfcatalase. By P. Nicholls (introduced by D. Keilin). (*Molteno Institute, University of Cambridge*)

Sulfhaemoglobin is formed during the action of hydrogen sulphide on oxyhaemoglobin. Previous investigations (Michel, 1938; Nijveld, 1943) have left the mechanism of formation and the structure of this derivative uncertain. The formation and properties of sulfmyoglobin have now been studied, and an analogous catalase compound, sulfcatalase, has been found.

Sulfmyoglobin is formed stoicheiometrically by the action of hydrogen sulphide on metmyoglobin peroxide. If the peroxide compound is written as ferrylmyoglobin (George & Irvine, 1955) the reaction may be formulated:

$$MbFeO^{2+} + H_2S \rightarrow \begin{cases} MbSFe^{2+} + H_2O & (1) \\ MbSFe^{3+} + H^{\cdot} + OH^{-} & (2) \end{cases}$$

The metsulfmyoglobin in (2) may be reduced to sulfmyoglobin by excess sulphide.

Sulfmyoglobin combines reversibly with CO, but not O_2; it is oxidized to metsulfmyoglobin by ferricyanide. Metsulfmyoglobin is similar to metmyoglobin, but the absorption bands are shifted 40–80 mμ towards the red. It combines with fluoride, cyanide, azide, and hydrogen peroxide; it has acid and alkaline forms, with a pK between 8·5 and 9·0. Sulfmyoglobin is converted to oxymyoglobin by hydrogen peroxide; metsulfmyoglobin to cyanmyoglobin by cyanide and dithionite.

Ferrosulfcatalase is formed by the action of hydrogen sulphide on catalase compound II:

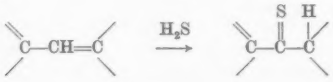
$$\text{Compound II} + H_2S \rightarrow \text{Catalase}\cdot S\cdot Fe^{2+} + H_2O. \quad (3)$$

It combines with carbon monoxide, which shifts its absorption maximum from 635 to 627 mμ. It is oxidized to sulfcatalase (ferric) by oxygen, but not by ferricyanide. Sulfcatalase and its derivatives

have absorption bands shifted 90 mμ towards the red, compared with normal catalase.

Sulfcatalase cannot be reduced to ferrosulfcatalase. It combines with fluoride, cyanide, and sulphide. Ethyl hydroperoxide gives normal compound II. Sodium dithionite reforms some catalase from both ferrous and ferric sulfcatalase.

The results indicate that sulf-compounds involve a reversible addition of sulphur to the porphyrin ring, possibly a thioketone structure at a methene bridge.



(4)

The occurrence of ferrosulfcatalase may account for the reported reduction of catalase by sulphide followed by dithionite (Zeile, Fawaz & Ellis, 1940); and for the light-sensitive CO inhibition of catalase in presence of thiols (which can generate hydrogen sulphide slowly) obtained by Keilin & Hartree (1938).

The analogous behaviour of metmyoglobin peroxide and catalase compound II towards hydrogen sulphide is further evidence of their chemical similarity. The formation of metsulfmyoglobin peroxide, and the absence of peroxide compounds of sulfcatalase, however, argue against identity of chemical structure.

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The Metabolism of [^{14}C]Chlorobenzene in the Locust. By TERESA KIKAL and J. N. SMITH. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W.* 2)

The fate of chlorobenzene in locusts (*Schistocerca gregaria*) has been studied with ring-labelled chlorobenzene. About half of an injected dose (0·05–0·5 mg./locust) is eliminated unchanged in 48 hr.

The greater part of the remaining activity is found in the excreta after 48 hr. Compounds identified by dilution analysis in excreta and locust tissues are *m*- and *p*-chlorophenols, 4-chlorocatechol, *p*-chlorophenylmercapturic acid and *p*-chlorophenylcysteine.

The three phenols each represent about 10% of the dose and the sulphur-containing metabolites a further 5%.

Only small amounts of *p*-chlorophenylmercapturic acid were found but it is possible that hydrolysis to *p*-chlorophenylcysteine occurs in the intestine of the locust after formation.

We are indebted to the Anti-Locust Research Centre for an expense grant for this work and for a maintenance grant to one of us.

Specificities of Ribonucleases in Liver Mitochondrial Fractions. By J. T. NODES and E. REID. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W.* 3)

Mitochondrial fractions from rat liver are known to contain both an acid and an alkaline ribonuclease. Supernatant fractions also contain an alkaline ribonuclease (possibly identical with that in mitochondrial fractions), together with an excess of inhibitor which normally masks the activity (Roth, 1957); but in the present study of specificities, only the ribonucleases of mitochondrial fractions have been examined.

Attempts to purify the two enzymes by methods such as those of Roth (1957) did not give satisfactory separations of the two activities. However, a solution of alkaline ribonuclease free of acid ribonuclease activity was obtained by heating the debris from a freeze-thawed mitochondrial fraction (cf. de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) for 10 min. at 65° at pH 3·5, followed by treatment at 0° with 0·25N-sulphuric acid. The solution remaining after sedimentation of the debris from the freeze-thawed mitochondrial fractions contained acid ribonuclease essentially free of alkaline ribonuclease. The former preparation, tested at pH 7·8, attacked liver ribonucleic acid (RNA) more readily than commercial RNA ('degraded'?) from yeast, and was inactive against the purine nucleotide 'core' remaining after the exhaustive digestion of liver or yeast RNA by pancreatic ribonuclease. The acid ribonuclease preparation, tested at pH 5·0, readily attacked yeast RNA or 'core' RNA.

Data obtained by paper chromatography indicated that acid ribonuclease is capable of splitting all phosphate linkages in the RNA molecule, whereas alkaline ribonuclease (in common with

pancreatic ribonuclease) attacks only between pyrimidine nucleotide residues. Neither enzyme preparation gave rise to nucleoside 5′-phosphates. Since cyclic nucleoside phosphates were obtained as reaction products at least with acid ribonuclease, cyclic adenosine and cytidine phosphates were tested as substrates. The alkaline ribonuclease preparation was inactive towards both, whereas the acid ribonuclease was active towards both, producing adenosine 2′-phosphate from cyclic adenosine phosphate and cytidine 3′-phosphate from cyclic cytidine phosphate. A paper-chromatographic assay method was developed for the reaction using cyclic substrates, and pH curves were obtained which confirmed that only the acid ribonuclease attacks cyclic nucleoside phosphates.

The specificity data now obtained are in accord with observations made independently by Zytko, de Lamirande, Allard & Cantero (1958). The possibility that more than one acid ribonuclease is present in the 'acid ribonuclease' preparation is now being studied.

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Metabolism of Rat-Liver Uridine Nucleotides after Adrenalectomy. By Barbara M. Stevens* and E. Reid. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W. 3*)

Reid & Stevens (1957) found abnormally low amounts of uridine mono-, di- ahd tri-phosphates (UMP, UDP, UTP) and of other uridine nucleotides in the livers of adrenalectomized rats 2–3 weeks after operation, but not (according to a preliminary experiment) 3 days after operation. Further experiments have now shown that at 3 days there may even be increased amounts, notably with UDP-glucose (about 60% increase). Since data for uridine nucleotide labelling after injection of ^{14}C-labelled orotic acid did not unequivocally establish that changes in levels were attributable to changes in the rates of synthetic steps, a number of *in vitro* experiments were performed, with cytoplasmic fractions prepared in 0·25M-sucrose solution.

Preliminary experiments to test the ability of such fractions to phosphorylate UMP to UDP and UTP showed that this ability was unimpaired 2–3 weeks after adrenalectomy. In subsequent experiments, based on those of Herbert, Potter & Hecht (1957), liver cytoplasm (freed from mitochondria) and labelled orotic acid were incubated with a glycolytic energy source to give labelled uridine nucleotides which were separated by gradient elution chromatography with Dowex 1 resin. The only marked and consistent effect of adrenalectomy was a decrease, especially at 2–3 weeks after operation, in the labelling of a fraction containing UDP-acetylglucosamine and UDP-glucose in admixture. Studies were made, with supernatant fractions, of the conversion of UTP to UDP-glucose (Maley & Lardy, 1956) and of UDP-glucose to UDP-glucuronic acid (Strominger, Maxwell, Axelrod & Kalckar, 1957), but no consistent changes in rate were found at any time after adrenalectomy. Evidently the *in vitro* findings so far obtained do not explain the changes in uridine nucleotide levels after adrenalectomy. It is possible that adrenalectomy affects a key step in the formation of orotic acid itself, the endogenous level of which is unfortunately too low to be measured.

In the *in vitro* experiments with orotic acid, measurements were made of the labelling of ribonucleic acid (RNA) as well as of nucleotides. With rats adrenalectomized 2–3 weeks previously, the labelling of cytoplasmic RNA tended to increase. This accords with the possibility suggested by *in vivo* experiments (Reid & Stevens, 1957) that one step affected by adrenalectomy is the formation of RNA from nucleotides.

* Gordon Jacobs Fellow of the Royal Marsden Hospital.

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The Cytase Activity of the Digestive Enzymes of *Helix pomatia* and the Purification of a Cellulase Component. By FAY L. MYERS and D. H. NORTHCOTE. (*Department of Biochemistry, University of Cambridge*)

A quantitative examination of enzymes present in a soluble extract prepared from the snail digestive tract has been made. These included invertase, lactase, maltase, cellobiase, xylanases, amylases, chitinase, cellulase, mannanases, glucanase and laminarinase. Their activities were compared at their pH optima by measurements of the amounts of reducing sugars formed. Cellofas B, a soluble cellulose derivative, was the most rapidly degraded carbohydrate and the disaccharides were broken down more slowly than the soluble polysaccharides. Lipase was measured on Tween 20, and a weak proteolytic activity was also investigated.

The extract has been used during an examination of the isolated cell wall of *Chlorella pyrenoidosa*. After a 14 hr. incubation, 21·1 % (w/w) of the wall material was digested away and this loss represented 70 % of the total α-cellulose, 43 % of the lipid and only 13 % of the hemicellulose of the wall. A microscopical examination of the material before and after digestion indicated the distribution of these components in the wall.

One of the cellulolytic enzymes has been purified by lead acetate precipitation (Keilin, 1956) followed by chromatographic separations on cellulose powder and Amberlite IRC 50 resin columns. Three protein fractions were eluted from the latter column and the second of these degraded both Cellofas B and phosphoric acid-swollen cotton linters (Walseth, 1952). This purified enzyme had optimum activity at pH 5·6, was denatured by heating above 60° or cooling below −15° and was reversibly inactivated in the presence of ammonium sulphate solutions. It degraded cellulose with the production of glucose and although not completely specific to cellulose, the enzyme did not attack β-methyl cellobioside or xylans. The activity of the enzyme towards both soluble and insoluble cellulose compounds was increased by the addition of enzymically inert protein. This added protein protected the enzyme from denaturation and did not have the effect on the substrate which Basu & Whitaker (1953) reported, using a fungal cellulase preparation.

One of us (F.L.M.) is indebted to the Agricultural Research Council for a grant.

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An Unusual Property of Thyroxine and other Iodophenols. By J. R. TATA.* (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

When a solution of ^{131}I-labelled L-thyroxine in an organic solvent (20–50 % propane-1:2-diol or *n*-butanol) is mixed with a large volume of aqueous buffer, there is an instantaneous loss of radioactivity in the thyroxine fraction; the residual radioactivity appears in the iodide fraction as seen by paper chromatographic analysis. This apparent deiodination is only transient; on aerobic incubation of the aqueous solution, without any other intervention, there is a spontaneous re-incorporation of radioactivity into the thyroxine fraction. The extent and rate of both breakdown and recombination are principally dependent upon the pH of the aqueous medium and the presence of light; concentration of thyroxine (within the limits of solubility) and temperature have little influence. Similar effects are also observed with the following iodophenols in decreasing orders: 3:5-diiodo-*p*-cresol, 3:5:3′-triiodo-L-thyronine, 3:5-diiodo-L-tyrosine, 3-iodo-L-tyrosine and 3-iodo-5-bromo-L-tyrosine. A relationship exists between the pH dependence of the initial breakdown and the apparent p*K* value for the phenolic OH of each iodophenol. It appears that the spontaneous disappearance results from the dissociation of the phenolic OH during the passage of the iodophenol from the organic solvent to the aqueous medium. This is substantiated by the abolition or reversal of 'deiodination' by acidification of the aqueous medium or by the addition of organic solvents. The initial breakdown is substantially retarded in the absence of light. The cause of the spontaneous reconstitution of radioactive iodophenols is unknown as is also the nature of the products formed. Although chromatographic analysis indicates the formation of iodide as the breakdown product, other methods do not reveal the presence of iodide. This property of iodophenols is not an artifact of radioactive exchange of iodine atoms and the re-formation of radioactive thyroxine leads to a true increase in its concentration as measured simultaneously by absorption in ultraviolet light and by chromatographic analysis.

* Beit Memorial Fellow for Medical Research.

Purification of Factor I of the Anthrax Toxin Produced *in vivo*. By J. L. STANLEY and H. SMITH. (*Microbiological Research Establishment, Porton, Wilts*)

The specific, lethal and oedema-producing toxin of *Bacillus anthracis*, found in the plasma of guinea pigs dying of anthrax (Smith, Keppie & Stanley, 1955) has at least two components which form a synergistic mixture (Smith *et al.* 1956). Factor I was deposited quickly by ultracentrifugation (leaving factor II in the supernatant); it was assayed by noting the first descending serial dilution which did not produce an oedematous skin reaction after being mixed with a fixed (and separately unreactive) amount of factor II.

Purification of factor I was considerably hampered by its extreme lability. The problem was to devise a fractionation which preserved the toxicity until a product showing some evidence for homogeneity was obtained; a 'toxoided' product, retaining its immunogenicity had already been prepared (Smith & Gallop, 1956). The toxic plasma used in the following process must be collected from guinea pigs which had died from anthrax only a few minutes previously. If the collection of plasma was delayed, factor I activity disappeared rapidly during the subsequent fractionation.

Crude factor I (10 ml., approx. 6 %, w/v, protein), prepared by a modification of the method of Smith *et al.* (1956), was precipitated 4 times with 25 % (w/v) ammonium sulphate at −8°. The final precipitate, containing 50–100 % of the original activity was dissolved in 0·005 *I* phosphate buffer (pH 7·4) and the solution (90 ml., approx. 0·2 %, w/v, protein) applied to a diethylaminoethylcellulose column (2 g., see next paper) at 2°; adsorbed factor I was eluted with 0·2 *I* phosphate buffer (pH 7·2) containing 0·2 M-NaCl. 50–100 % of the initial activity was in the active fraction and 5–7 % of the original protein. Factor I was finally sedimented at 100 000 *g* and at 2° without significant loss of activity.

The final product (1–2 mg.) was very labile especially at pH values removed from neutrality; although essentially homogeneous in the ultracentrifuge at pH 7·4 it readily aggregated and disaggregated with loss of toxicity. The product remained as one band on electrophoresis on paper in 0·05 *I* phosphate buffer at pH 7·4 for 24 hr. at 2°, and it (50 μg.) produced one line in serological precipitation plates against anthrax antiserum. It had nitrogen, 11·4 %; ash (sulphated), 6·2 %; carbohydrate 4·6 % (calc. as glucose); and lipid, at least 13 %.

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Purification of Factor II of the Anthrax Toxin Produced *in vivo* using Cellulose Ion-Exchange Columns. By K. SARGEANT and H. SMITH. (*Microbiological Research Establishment, Porton, Wilts*)

On ultracentrifugation of crude anthrax toxin, factor II remained in the supernatant (see previous communication). It was assayed by determining the first descending serial dilution which did not give an oedematous skin reaction after being mixed with a fixed (and separately unreactive) amount of factor I. Like factor I, factor II was extremely labile, and only mild purification methods could be used if its potential toxicity was to be preserved. After the failure of most classical procedures in this respect, ion-exchange cellulose columns (Peterson & Sober, 1956) were used successfully.

Crude factor II, prepared by a modification of the method of Smith *et al.* (1956) was dialysed at 2° against a phosphate buffer (Na_2HPO_4, 4·26 g.; KH_2PO_4, 2·72 g.; NaCl, 2·77 g./l.) at pH 6·9. The dialysate (30 ml., approx. 2 %, w/v, protein) was applied at 2° to a column of diethylaminoethylcellulose (10 g.) previously equilibrated at pH 7·0 with a second phosphate buffer (Na_2HPO_4, 4·26 g.; KH_2PO_4, 2·72 g./l.). The column was eluted (7 ml./min.) with the original buffer until all the haemoglobin present was removed (vol. 50–75 ml., containing 30–45 % of the original activity). A single fraction (150 ml.) was then taken which contained 30–40 % of the original activity and approx. 10 % of the protein. The second fraction was dialysed (24 hr.) at 2° and freeze-dried without significant loss of activity.

On paper electrophoresis for 16 hr. in 0·2 *I* barbitone buffer at pH 8·6 and 2°, the freeze-dried material was separated into serum albumin and a minor component (approx. 10 %) of lower mobility. The albumin fraction was inactive but the minor component, when eluted, contained approx. 50 % of the original activity.

After dialysis and freeze-drying, this final product (2–5 mg.) sedimented as one component in the ultracentrifuge at pH 7·7 and on paper electrophoresis under the conditions previously described, it moved as one band 7 cm. towards the anode in 16 hr. (potential gradient 10 v/cm.). On serological precipitation plates against anthrax antiserum it (100 μg.) formed one line, different from that produced by factor I. It had nitrogen, 13·9 %; ash (sulphated), 1·6 %; carbohydrate, 3·7 % (calculated as glucose); phosphorus, less than 0·2 %.

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The Production of Pentose-Cycle Intermediates in Ordinary Growing Cultures of Saprophytic Soil Nocardias and Mycobacteria. By R. B. Duff and D. M. Webley (introduced by J. S. D. Bacon). (*The Macaulay Institute for Soil Research, Aberdeen, Scotland*)

The H.M.P. shunt in micro-organisms has hitherto been established by demonstrating that the appropriate enzymes exist in the cell-free extracts (e.g. see Dickens, 1956). Other workers (Mortenson & Wilson, 1955; Ghiretti & Guzman Barron, 1954; Godin, 1953) have shown that rather small amounts of the intermediates can be detected in the cells or the culture fluid.

Our organism, a paraffin- and fat-decomposing soil nocardia (*N. opaca* strain T_{16}, Webley, 1954), when grown on certain substrates (glucose, glycerol, mannitol, gluconic acid, sorbitol and fructose) liberates considerable amounts of ribulose, sedoheptulose and (usually) dihydroxyacetone into the liquid medium, in concentrations that can easily be seen on paper chromatograms. For characterization of the intermediates the organism was grown on a shaking machine on a medium containing 4 % (w/v) glycerol or glucose, mineral salts, calcium carbonate, and yeast extract or sodium glutamate. After 17 days cells were removed and the deionized supernatant concentrated and applied to a column of cellulose powder. From 150 ml. of medium we obtained fractions containing: dihydroxyacetone (3 g.), ribulose (99 mg.), ribulose + pentulose (110 mg.), pentulose (8 mg.), sedoheptulose + pentose (40 mg.), and sedoheptulose (190 mg.). The intermediates were characterized chromatographically and by other methods. The pentulose mentioned was neither ribulose nor xylulose but gave a spectrum similar to these in the cysteine–carbazole reaction (Ashwell & Hickman, 1957).

In small-scale growth experiments with glucose and glycerol as substrates we obtained as much as 3 and 1·3 mg./ml. ribulose and 1·5 and 1·3 mg./ml. sedoheptulose respectively (estimated on the paper chromatogram). In the absence of calcium carbonate there was less growth and the initial glucose concentration (40 mg./ml.) was reduced only to 26 mg./ml. in 8 days. With calcium carbonate added complete utilization resulted. The absence of calcium carbonate also resulted in the production of the intermediates at a much later stage and in smaller amounts. With sucrose only small amounts were produced at a late stage with calcium carbonate. Fructose was also present.

Out of five strains of mycobacteria and fifteen nocardias tested only two and six respectively produced the intermediates. The nocardias produced much more than the closely related mycobacteria. *Acetobacter suboxydans* (NCIB 5595) gave dihydroxyacetone with these conditions but not the other intermediates although enzymes of the H.M.P. shunt are present in strains of this organism (Hauge, King & Cheldelin, 1955).

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Aerobic Inhibition of Glycolysis in Brain Mitochondria. By R. BALAZS and D. RICHTER. (*Medical Research Council Neuropsychiatric Research Unit, Whitchurch Hospital, Cardiff*)

It was previously reported that aerobic inhibition of glycolysis in rat-brain mitochondria is due in part to a process operating below the fructose diphosphate (FDP) level in the glycolytic chain (Balazs & Richter, 1958). The point of inhibition has now been further investigated.

'Sucrose mitochondria' were incubated in a medium containing 0·005–0·01M-FDP, 0·01M-α-oxoglutarate, 0·002M-adenylic acid, 0·00033M-DPN, 0·006–0·01M-potassium phosphate buffer pH 7·4, 0·01M-tris 2-amino-2-(hydroxy-methyl)-propane-1:3-diol buffer pH 7·4, 0·006M-$MgSO_4$, 0·002M-ethylenediaminetetra-acetate and 0·025M-nicotinamide.

Under aerobic conditions lactate production was inhibited by 20–40%, but triose phosphate formation remained unchanged. Even after blocking the glyceraldehyde phosphate dehydrogenase with iodoacetate (0·003M) there was no difference in the aerobic and anaerobic formation of triosephosphate. Aldolase was therefore not involved in the inhibition.

Aerobic inhibition of lactate formation was also observed after blocking with fluoride (0·01M), in the presence of pyruvate (0·006M) to reoxidize $DPNH^+$ formed by the triose phosphate oxidation (Aisenberg & Potter, 1957). Since enolase and phosphoenolpyruvate kinase are excluded, the inhibition in this system could occur either at the oxidation of glyceraldehyde phosphate or of $DPNH^+$. In the first case, less phosphoglycerate would be formed under aerobic conditions: otherwise its formation would remain unchanged. It was found that both phosphoglycerate and lactate formation was inhibited to about the same extent under aerobic conditions. In a typical experiment the following values were obtained for lactate and phosphoglycerate (expressed as μmoles per 40 min. per mitochondria, equivalent to 600 mg. fresh tissue): lactate, aerobic 2·96, anaerobic 5·8; phosphoglycerate, aerobic 2·42, anaerobic 4·5.

Diphosphoglycerate phosphokinase was excluded by the finding that aerobic inhibition of lactate formation was not altered by the presence of arsenate (0·008M) and fluoride (0·01M).

Thus it is evident from these experiments that in brain mitochondria the aerobic inhibition of glycolysis below the FDP level occurs at the phosphoglyceraldehyde dehydrogenase step.

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Phosphorylation by Illuminated Chloroplast Preparations. By D. A. WALKER and R. HILL. (*Department of Biochemistry, University of Cambridge*)

Arnon, Whatley & Allen (1955) found that the phosphorylating system in chloroplast preparations required the addition of certain substances such as flavine mononucleotide (FMN) and 3-methyl 1:4-naphthoquinone (K_3) in order to show activity. We have examined a number of other reagents which also activate the system. Adenosine diphosphate, 5×10^{-3}M, was used as acceptor in presence of the same concentration of inorganic phosphate. The active reagents were used at a final concentration of 10^{-5}M; they were all capable of showing reversible reduction and oxidation (chemically) but the range of characteristic oxidation-reduction potential varied from E'_0, pH 7 −445 mv (methyl viologen) to +248 mv (naphthoquinone 4-sulphonate). While 2:6-dichlorophenolindophenol is inactive, dichlororesorufin gave the same order of activity as FMN. The very marked activity of phenazine methosulphate (noted by Jagendorf & Avron, 1958), was found to be due to its conversion into pyocyanine (McIlwain, 1937). In complete absence of oxygen phenazine methosulphate gave no activity unless it had previously been exposed in solution to light and air. With freshly purified pyocyanine the concentration required to develop half activity was $6{\cdot}3 \times 10^{-6}$M, as compared with 5×10^{-7}M for FMN and K_3. The pH-activity curve obtained with pyocyanine showed two maxima, a higher one at pH 7 and a shoulder at pH 7·7. Corresponding curves with FMN and K_3 show a single maximum at pH 7·7 which is lower than that given by pyocyanine. With aged chloroplast preparations pyocyanine gave only a single maximum at pH 7·7. This might be taken as evidence that more than one phosphorylation step can result from the action of light and that one step is readily lost on storage. If so, the chloroplast system presents a problem similar to that arising from the study of respiratory chain phosphorylation in mitochondria (Slater, 1956).

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The Effect of Sodium Salicylate on the Oxygen Consumption of Normal Human Leucocytes *in vitro*. By SHEENAH K. BISSET and W. D. ALEXANDER. (*Biochemistry Department and Clinical Chemotherapeutic Research Unit of the Medical Research Council, Western Infirmary, Glasgow*)

Since the leucocyte is an easily obtainable, free, nucleated cell, containing the enzymes for glycolysis and respiration, it may be suitable for the study of cellular metabolic activity in man. We have investigated the effect of the metabolic stimulant, sodium salicylate, on the oxygen consumption of this intact cell in order to compare results with other *in vitro* and *in vivo* studies of salicylate on oxygen consumption (Sproull, 1954; Alexander & Johnson, 1958; Reid, 1957).

The leucocytes were obtained by sedimentation from heparinized blood (300 ml.). The donors were eight healthy medical students. Total and differential white cell counts were all found to be within the normal range. Sufficient leucocytes were obtained from 300 ml. of blood to study the effect of sodium salicylate over the range 10–160 mg. per 100 ml. medium. The number of leucocytes per flask was regulated to about 50×10^6. The medium used was a modified Krebs's no. 2 buffer and oxygen consumption was measured by the direct method in Warburg respirometers at 37°. Oxygen consumption was linear for at least 3 hr.

Between 10 and 40 mg. sodium salicylate per 100 ml. medium, the oxygen consumption increased in proportion to the sodium salicylate concentration; the maximum stimulation occurred at a concentration of 40 mg. per 100 ml., and thereafter up to 80 mg. per 100 ml., the stimulation decreased; between 80 and 160 mg. per 100 ml., oxygen consumption was progressively depressed.

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The Relation of Diaphorase and Cytochrome Reductase. By V. MASSEY. (*Department of Biochemistry, University of Sheffield*)

It has been suggested (Mahler, 1955) that DPNH diaphorase prepared from heart muscle by the method of Straub (1939) is a degraded form of DPNH cytochrome *c* reductase, differing from the latter in having lost its iron atoms, and hence its capacity for reducing cytochrome *c*. Evidence that diaphorase is in fact an undegraded enzyme, not related to cytochrome reductase, has been obtained by differential extraction of the two enzymes from pig-heart muscle under a variety of conditions. Diaphorase can be extracted at 4° in the presence of 1% (w/v) ammonium sulphate in the same yield as obtained in the normal heat extraction under these conditions. The cold extract was practically free of cytochrome *c* reductase; on heating at 38° this enzyme was extracted with the diaphorase. Quantitative separation of the two enzymes in any extract in which they occur together, and considerable purification can be achieved very simply by differential elution from a calcium phosphate gel-cellulose column.

The fractionation of diaphorase was also examined in the ethanolic extraction of cytochrome reductase by the method of Mahler, Sarkar, Vernon & Alberty (1952). By this method, the extract of cytochrome reductase, while possessing diaphorase-like activity, was completely free of Straub-diaphorase. The latter enzyme could be extracted in good yield and purified from the insoluble residue left after the extraction of cytochrome reductase.

The most compelling evidence against the concept of diaphorase being a degraded form of cytochrome reductase comes from the fact that after extraction of diaphorase at 4° with 1% ammonium sulphate, cytochrome reductase can then be obtained in excellent yield by ethanolic extraction of the residue from the diaphorase extraction.

The differential extraction of the two enzymes using mild conditions makes it extremely unlikely that diaphorase is a modified form of cytochrome reductase, and again opens up the question of the physiological role of diaphorase. A possible

function of the enzyme is to couple the oxidation of DPNH and the reduction of cytochrome *c* employing a small molecular weight electron carrier as intermediate between the large molecules of cytochrome *c* and diaphorase. Of a number of biological redox systems tested, α-lipoic acid was found to act as an efficient coupling agent linking oxidation of DPNH by diaphorase to reduction of cytochrome *c*.

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The Effect of Human Serum Protein Fractions on the Glucose Uptake of the Isolated Rat Diaphragm. By K. W. Taylor. (*Department of Biochemistry, University of Cambridge*)

Stimulation of the glucose uptake of the isolated rat diaphragm has been used extensively as the basis of an *in vitro* procedure to assay insulin. A difficulty in applying this method to the assay of insulin in plasma is the possibility that substances in plasma other than insulin may affect the glucose uptake of the diaphragm. In an attempt to separate the effects of insulin from those of other substances serum proteins have been fractionated by zone electrophoresis on columns of 'ethanolysed' cellulose using a phosphate borate buffer (Campbell & Stone, 1956). Following elution from the column, fractions were dialysed against distilled water, and lyophylized. The dried fractions were dissolved in a bicarbonate buffered saline solution (Gey & Gey, 1936) and their influence on the uptake of glucose by isolated rat diaphragm investigated.

Preliminary experiments showed that when an excess of ^{131}I-labelled insulin is added to human serum, and the mixture electrophoresed, a peak of radioactivity is associated with the α_1-globulins and slower moving albumin fractions. Maximal biological activity is also associated with the same slower moving albumin zone after the electrophoresis of serum mixed with an excess of unlabelled insulin. When normal human serum without any additions is subject to electrophoresis, maximal activity also appears in the α_1-globulin and albumin zone. The β- and γ-globulins obtained under these conditions also stimulate the uptake of glucose by the diaphragm and this effect may also be due to insulin.

Since serum albumin fractions may be obtained both from human and from ox sera, which do not affect the glucose uptake of the diaphragm, it is inferred that albumin itself is without influence on glucose uptake under the conditions of these experiments. In a further series of experiments, it has been shown that insulin added to human or rat serum in low concentration is co-precipitated with albumin, when this is prepared by the method of Korner & Debro (1956). It is concluded that the stimulating effect of such albumin preparations (Randle, 1957) on the uptake of glucose by diaphragm is due to the contamination of these albumin preparations by serum insulin.

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Isolation and Structure of the Fluorescent Compound Formed from Adrenaline and Ethylenediamine. By J. Harley-Mason and A. H. Laird. (*Chemical Laboratory, University of Cambridge*)

A method for the determination of adrenaline in biological fluids involving oxidative reaction with ethylenediamine to give a stable yellow fluorescent product has been described by Weil-Malherbe & Bone (1952, 1954, 1957). We have now isolated this product in pure form by reaction of adrenochrome with ethylenediamine in aqueous methanol and have shown its structure to be dihydro-3-hydroxy-1-methylpyrrolo-[4:5-g]-quinoxaline. This structure has the same ring system as that originally suggested by Weil-Malherbe & Bone (1952), but contains one more double bond and is thus not derived by simple condensation of adrenochrome with ethylenediamine, but requires an additional oxidative step. Under the conditions we used, the reaction is far from quantitative and several coloured—some of them fluorescent—by-products are obtained.

Weil-Malherbe & Bone (1952) further noted that oxidative reaction of catechol with ethylenediamine gave a yellow fluorescent product. We have also isolated this material in pure form and shown

it to be 1:2:3:4-tetrahydro-1:4:5:8-tetra-aza-anthracene. The yield in this case is also poor, but we have found that the material can conveniently be prepared in quantity by the reaction of 2:5-dihydroxy-*p*-benzoquinone with ethylenediamine in aqueous solution in the presence of air. The chemistry of these fluorescent materials and the mechanism of their formation will be discussed.

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Use of *p*-Dimethylaminocinnamaldehyde as a Spray Reagent for Indole Derivatives on Paper Chromatograms. By J. HARLEY-MASON and A. A. P. G. ARCHER. (*Chemical Laboratory, University of Cambridge*)

A solution of *p*-dimethylaminobenzaldehyde in acid (Ehrlich's reagent) has long been used as a colour reagent for indoles and pyrroles. We have now examined the use of *p*-dimethylaminocinnamaldehyde* (DMCA) for this purpose. DMCA is a vinylogue of Ehrlich's reagent and might therefore be expected to give more intensely coloured condensation products owing to greater conjugation.

The spray reagent was made up as follows: DMCA (2 g.) was dissolved in 6N-HCl (100 ml.) and ethanol (100 ml.). Trials were conducted on Whatman no. 1 paper in the usual manner. Intensely coloured spots were obtained using a variety of indoles including indole (dark green), 2-methylindole (khaki), skatole (violet), tryptophan and indole-3-acetic acid (purple) and 5-hydroxytryptamine (blue). Moreover, a comparative test with standard Ehrlich's reagent using indole and tryptophan showed that the DMCA reagent is approximately ten times as sensitive as a detector. However, DMCA is more reactive, and therefore less selective, than Ehrlich's reagent and its reactions with other groups of compounds will be discussed.

* Available from the Aldrich Chemical Company, 3747 N. Booth Street, Milwaukee 12, Wisconsin, U.S.A.

Further Studies of Vitamin B_{12} Uptake by Rat Intestine. By A. L. LATNER, C. GREEN and LAUREEN RAINE. (*Department of Pathology, King's College, University of Durham, in the Royal Victoria Infirmary, Newcastle upon Tyne*)

The isolated upper small intestine of the rat has a 'competitive' and a 'non-competitive' system for vitamin B_{12} uptake (Latner & Raine, 1958). These studies have now been extended to intact rats, fed radioactive vitamin B_{12} alone or mixed with an analogue and killed at varying times after oral dosage up to a maximum of 5 hr. The small intestine was removed and the total radioactivity of contents, mucosal layer and submucosal layers, separately determined. Uptake by the submucosal layers was almost negligible. Mucosal uptake with the vitamin alone showed a peak at 1 hr.; with pseudo-vitamin B_{12} a plateau occurred at 30–105 min. and the level at 1 hr. was significantly lowered (10 paired observations; $t = 4{\cdot}1$, $P < 0{\cdot}001$) indicating two vitamin B_{12} binding systems similar to isolated intestine. This is an interesting finding in view of the fact that, unlike the experiments with isolated intestine, most of the vitamin B_{12} in the case of the intact rats was in the bound form before intestinal uptake. It was possible to correlate uptake with absorption and to confirm an initial absorption delay period (Booth, Chanarin, Anderson & Mollin, 1957) and that uptake equates with absorption.

Since maximum uptake occurs in the middle of the small intestine (Booth *et al.* 1957; Latner & Raine, 1957), these studies have been extended by dividing it into quarters after previous ligation at 6–8 cm. intervals to prevent mixing of the contents. The third quarter showed maximum uptake but a significant amount occurred in the second. Uptake was depressed by pseudovitamin B_{12} and markedly depressed by anti-vitamin B_{12} (mixture of monoethylamides).

The experiments with strips of isolated upper small intestine (Latner & Raine, 1958) have been repeated using 4 cm. portions from the region of

greatest uptake. The results were very similar to those already reported but plateau levels were somewhat higher.

The concept of two vitamin B_{12} binding systems can now be extended to the intact animal as well as to the portion of intestine most active in the absorption mechanism. The suggestion is tentatively put forward that the 'competitive' system might act as a barrier to absorption, which is overcome by the action of intrinsic factor.

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Sex Differences in the α-Tocopherol Requirement of Rats as Indicated by the Haemolysis Test. By R. J. Ward. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

Experience in this laboratory has indicated that when equal amounts of α-tocopherol are given to adult rats, the erythrocytes of the females are protected for a longer period than those of males against the haemolytic action of dialuric acid (Gyorgy & Rose, 1949). In 1950, Rose & Gyorgy reported that a daily intake of 3 mg. of DL-α-tocopherol per kg. of body weight was sufficient to protect the erythrocytes, but no mention was made of the sex of the animals. Recently, Taylor (1958) reported that in rats deficient in vitamin E, haemolysis was greater in males than females when they were exposed to abnormally high pressures of oxygen. It was of interest to ascertain whether the apparent larger requirement of tocopherol by the male is due only to the larger size of the animal, or to a sex difference in the requirement of vitamin E. From weaning, groups of two male and two female rats were given a standard diet, deficient in vitamin E, with weekly supplements of vitamins A, D and K. α-Tocopherol was given in two equal doses at 2·0, 2·5 or 3·0 mg./kg. body weight weekly from the 42nd day of the experiment. At 18 weeks, the mean percentage haemolysis of the erythrocytes, 48 hr. after dosing, was 80, 60 and 33 for the males of mean weight 357 g. and 53, 4 and 3 for the females of mean weight 238 g. for the three levels of intake respectively. At 39 weeks, 72 hr. after dosing, the mean percentage haemolysis was 87, 92 and 39 for the males of mean weight 369 g. and 98, 2 and 7 for the females of mean weight 227 g. for the three levels of intake respectively. In a confirmatory experiment, α-tocopherol was administered to weanling rats as a single weekly dose at levels of 2·0, 2·5, 3·0 and 3·5 mg. per kg. body weight. In the sixth week of the experiment, 48 hr. after the weekly dose, when the mean weight of the males was 281 g. and the females 188 g., the mean percentages of haemolysis in the males were 95·9, 88·0, 70·2 and 34·8 and in the females 14·7, 6·3, 11·1 and 1·6 for the four levels of intake respectively. It is therefore concluded that a genuine sex difference exists.

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The Partial Vitamin E Activity of *NN'*-Diphenyl-*p*-phenylenediamine in the Rat. By I. M. Sharman and T. Moore. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

Experiments by several workers (Singsen *et al.* 1955; Jensen, Carver & McGinnis, 1956; Ames, Ludwig, Swanson & Harris, 1956; Draper, Goodyear, Barbee & Johnson, 1956, 1958) have shown that the well-known commercial anti-oxidant, *NN'*-diphenyl-*p*-phenylenediamine (DPPD) may be added to the list of artificial substances, including methylene blue (Dam, Kruse, Prange & Sondergaard, 1951; Moore, Sharman & Ward, 1953), which can act as partial substitutes for vitamin E. In experiments on rats we have found that DPPD not only equals methylene blue in preventing brown uterus, a typical sign of avitaminosis E, but can also prevent lesions against

which we have found methylene blue to be ineffective. Thus DPPD, when included as 0·125% of a standard diet deficient in vitamin E, prevented both degeneration of the testes and also the abnormal liability of the erythrocytes to haemolysis (Gyorgy & Rose, 1949).

In examination under ultraviolet irradiation of the body fat of rats which had received DPPD, we observed a bright purple fluorescence. Spectroscopic examination of the unsaponifiable fraction of the fat revealed a sharp band at about 300 mμ, the maximum for DPPD. It seems probable that the apparent superiority of DPPD over methylene blue as a vitamin E substitute is due to its ability, shared by natural vitamin E, of being absorbed into the body fat.

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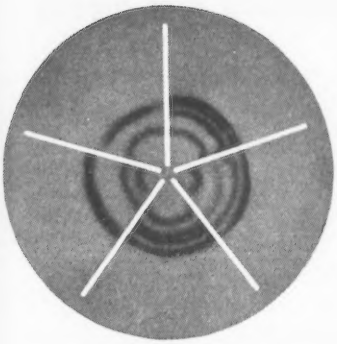

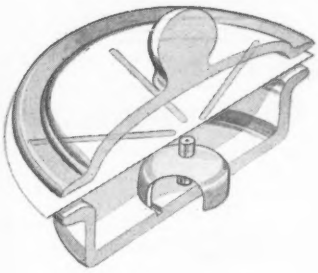

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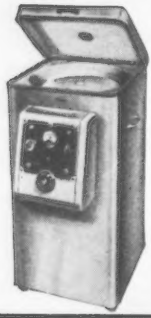

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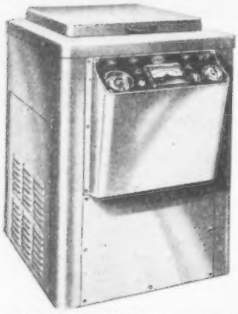

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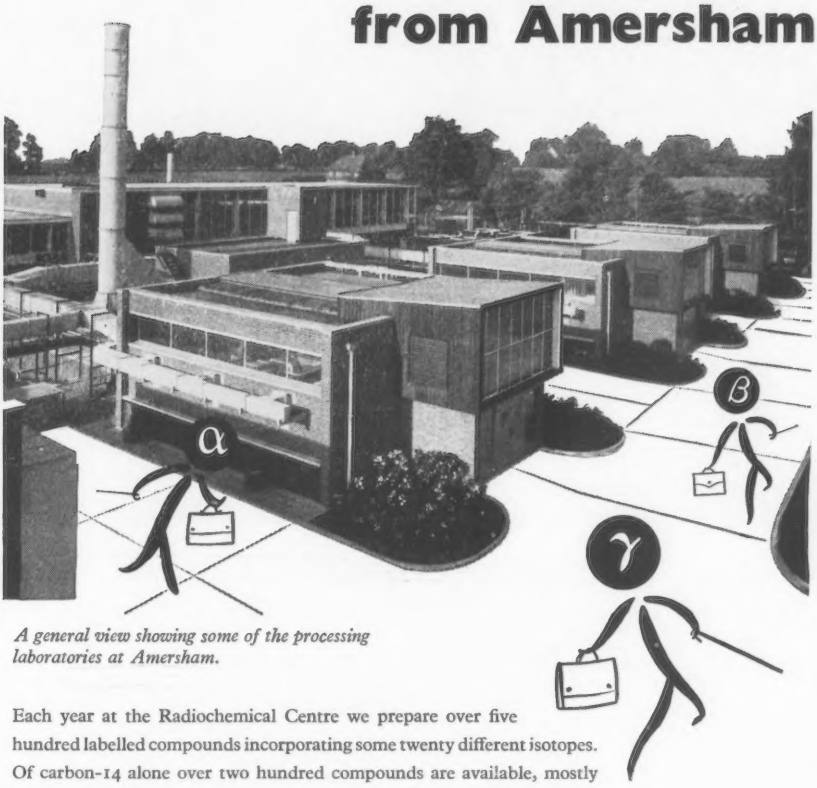

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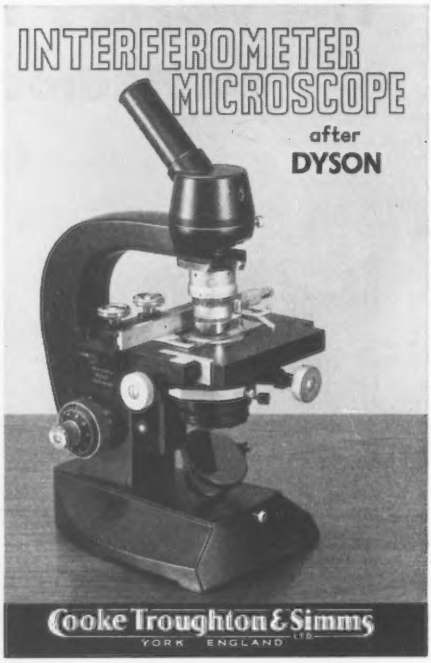

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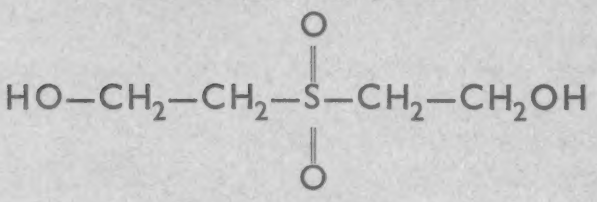

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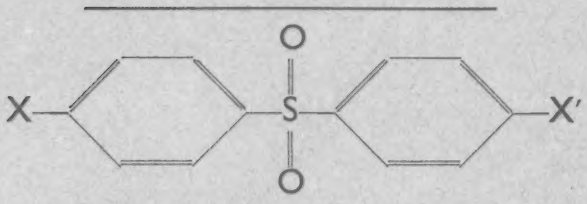

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